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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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VOLUME LXXI
AUGUST TO DECEMBER, 1936

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Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Mass., between June 1 and October 1 and to the Institute of Biology, Divinity Avenue, Cambridge, Mass., during the remainder of the year.

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CONTENTS

No. 1. AUGUST, 1936

PAGE

THIRTY-EIGHTH REPORT OF THE MARINE BIOLOGICAL LABORATORY 1

WATERMAN, A. J.
The Membranes and Germinal Vesicle of the Egg of Sabellaria vulgaris 46

TYLER, ALBERT
On the Energetics of Differentiation, III 59

TYLER, ALBERT
On the Energetics of Differentiation, IV 82

HARVEY, ETHEL BROWNE
Parthenogenetic Merogony or Cleavage without Nuclei in Arbacia punctulata 101

COE, W. R.
Sequence of Functional Sexual Phases in Teredo 122

RAKESTRAW, NORRIS W.
The Occurrence and Significance of Nitrite in the Sea 133

FISH, CHARLES J.
The Biology of Oithona similis in the Gulf of Maine and Bay of Fundy 168

BEAMS, H. W., AND R. L. KING
The Effect of Ultracentrifuging upon Chick Embryonic Cells, with special reference to the "Resting" Nucleus and the Mitotic Spindle 188

DELAMATER, ARLENE JOHNSON
The Effects of Heavy Water upon the Fission Rate and the Life Cycle of the Ciliate, Uroleptus mobilis 199

YOUNG, ROBERT T., AND DENIS L. FOX
The Structure and Function of the Gut in Surf Perches (Embiotocidae) with reference to their Carotenoid Metabolism 217

METZ, C. W.
Effects of Mechanical Distortion on the Structure of Salivary Gland Chromosomes 238

GEORGE, W. C.
The Rôle of Blood Cells in Excretion in Ascidians 249

No 2. October, 1936

PAGE

Parker, G. H.
The Reactivation by Cutting of Severed Melanophore Nerves in the Dogfish Mustelus 255
Abramowitz, A. A.
Physiology of the Melanophore System in the Catfish, Ameiurus 259
Smith, G. M., and C. W. Coates
Cutaneous Melanosis in Lungfishes (Lepidosirenidae) 282
Cowles, R. P., and C. E. Brambel
A Study of the Environmental Conditions in a Bog Pond with Special Reference to the Diurnal Vertical Distribution of Gonyostomum semen 286
Heilbrunn, L. V.
Protein Lipid Binding in Protoplasm 299
Mazia Daniel, and Jean M. Clark
Free Calcium in the Action of Stimulating Agents on Elodea Cells 306
Zobell, Claude E., and D. Quentin Anderson
Observations on the Multiplication of Bacteria in Different Volumes of Stored Sea Water and the Influence of Oxygen Tension and Solid Surfaces 324
Trager, William
The Utilization of Solutes by Mosquito Larvae 343
Coe, W. R.
Environment and Sex in the Oviparous Oyster Ostrea virginica 353
Grafflin, Allan L.
Renal Function in Marine Teleosts. IV. The excretion of inorganic phosphate in the sculpin 360
Koonz, Carl H.
Some Unusual Cytological Phenomena in the Spermatogenesis of a Haploid Parthenogenetic Hymenopteran, Aenoplex smithii (Packard) 375
Program and Abstracts of Scientific Meetings, Marine Biological Laboratory, 1936 386

No. 3. December, 1936

Waterman, A. J.
The Effect of Alcohol on Gastrulation in Arbacia 413
Coonfield, B. R.
Regeneration in Mnemiopsis leidyi, Agassiz 421

PAGE

BOWEN, R. E.
Sensory Hairs of the Dogfish Ear 429

GIER, HERSCHEL T.
The Morphology and Behavior of the Intracellular Bacteroids of Roaches 433

SAXTON, JOHN A., JR., MARY M. SCHMECKEBIER AND ROBERT W. KELLEY
Autogenous Transplantations of Pigmented and Unpigmented Ear Skin in Guinea Pigs 453

GUYER, M. F., AND PEARL E. CLAUS
Recovery Changes in Transplanted Anterior Pituitary Cells Stratified in the Ultra-Centrifuge 462

CAROTHERS, E. ELEANOR
Components of the Mitotic Spindle with Especial Reference to the Chromosomal and Interzonal Fibers in the Acrididæ 469

LACKEY, JAMES B.
Some Fresh Water Protozoa with Blue Chromatophores 492

ERIKSSON-QUENSEL, INGA-BRITTA, AND THE SVEDBERG
The Molecular Weights and pH-Stability Regions of the Hemocyanins 498

INDEX 548

Volume LXXI Number 1

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INSTRUCTIONS TO AUTHORS

Preparation of Manuscript. In addition to the text matter, manuscripts should include a running page head of not more than thirty-five letters. Footnotes, tables, and legends for figures should be typed on separate sheets.

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Proof. Page proof will be furnished only upon special request. When cross-references are made in the text, the material referred to should be marked clearly on the galley proof in order that the proper page numbers may be supplied.

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BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

THIRTY-EIGHTH REPORT, FOR THE YEAR 1935—
FORTY-EIGHTH YEAR

I. TRUSTEES AND EXECUTIVE COMMITTEE (AS OF AUGUST 13 1935) 1
LIBRARY COMMITTEE 3
II. ACT OF INCORPORATION 3
III. BY-LAWS OF THE CORPORATION 4
IV. REPORT OF THE TREASURER 5
V. REPORT OF THE LIBRARIAN 9
VI. REPORT OF THE DIRECTOR 10
Statement 10
Addenda:
1. The Staff, 1935 14
2. Investigators and Students, 1935 16
3. Tabular View of Attendance 26
4. Subscribing and Coöperating Institutions, 1935 27
5. Evening Lectures, 1935 28
6. Shorter Scientific Papers, 1935 28
7. Members of the Corporation 33

I. TRUSTEES

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II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 11.30 A.M., daylight saving time, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio,* who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeritus shall have all rights of the Trustees except that Trustees Emeritus shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. Inasmuch as the time and place of the Annual Meeting of Members is fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of said meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen: Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1935.

The accounts have been audited by Messrs. Seaman, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

At the end of the year 1935, the book value of the Endowment Funds in the hands of the Central Hanover Bank and Trust Company as Trustee, was

General Fund,	Securities	$ 884,683.36
	Cash	26,482.57
Library Fund,	Securities	193,582.06
	Cash	101.19
Total book value		$1,104,849.18

The income collected from these Funds was as follows:

General Endowment	$40,479.31
Library Fund	7,471.45
	$47,950.76

an increase of a little more than one thousand dollars ($1,000) over the income from these Funds in 1934.

The income in arrears, some of which may never be collected, was on December 31, 1935:

General Fund	$13,944.05
Library Fund	4,150.00

The extra dividends from the stock of the General Biological Supply

House have continued; the total dividends received from that Company amounted during the year to $10,922.00.

Retirement Fund. A total of $4,560 in pensions and benefits was paid out of the Retirement Fund. The Fund at the end of the year consisted of securities of the book value of $21,129.42
Cash 48.37

Total $21,177.79
Income in arrears on December 31 was $321.98

The land, buildings, equipment and library (exclusive of the Gansett and Devil's Lane tracts) represented an investment of ... $1,727,703.99
less reserve for depreciation of 445,913.96

$1,281,790.03

Expenses including $43,625.42 reserve for depreciation exceeded income by $14,794.20. There was expended from current funds $22,087.34 for plant account, mostly for equipment and books.

The property of the Laboratory is free and clear of mortgages. At the end of the year it owed on open account $4,427.07 and had accounts receivable of $7,345.83 and $13,541.05 in cash and bank accounts.

Following is the balance sheet as of December 31, 1935, the condensed statement of income and outgo, and the surplus account, all as set out by the accountants.

EXHIBIT A

MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1935

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee—Schedules I-a and I-b		$1,104,849.18	
Securities and Cash—Minor Funds—Schedule II		8,108.24	$1,112,957.42
Plants Assets:			
Land—Schedule IV	$ 98,103.05		
Buildings—Schedule IV	1,226,910.53		
Equipment—Schedule IV	167,022.12		
Library—Schedule IV	235,668.29	$1,727,703.99	
Less Reserve for Depreciation		445,913.96	
		$1,281,790.03	
Cash in Dormitory Building Fund		358.24	
Cash in Reserve Fund		24.65	$1,282,172.92

Current Assets:			
Cash		$ 13,541.05	
Accounts and Notes-Receivable		7,345.83	
Inventories			
Supply Department	$ 38,399.27		
Biological Bulletin	11,384.66	49,783.93	
Investments:			
Devil's Lane Property	$ 43,341.07		
Gansett Property	5,511.29		
Stock in General Biological Supply House, Inc.	12,700.00		
Securities and Cash—Retirement Fund—Schedule V	21,177.79	82,730.15	
Prepaid Insurance		3,126.19	
Items in Suspense (Net)		146.30	$ 156,673.45
			$2,551,803.79

Liabilities

Endowment Funds:		
Endowment Funds—Schedule III	$1,104,849.18	
Minor Funds—Schedule III	8,108.24	$1,112,957.42
Plant Funds:		
Donations and Gifts—Schedule III	$1,029,572.61	
Other Investments in Plant from Gifts and Current Funds	252,600.31	$1,282,172.92
Current Liabilities and Surplus:		
Accounts—Payable	$ 3,763.15	
Woods Hole Oceanographic Institution	663.92	
	$ 4,427.07	
Current Surplus—Exhibit C	152,246.38	$ 156,673.45
		$2,551,803.79

EXHIBIT B

MARINE BIOLOGICAL LABORATORY INCOME AND EXPENSE, YEAR ENDED DECEMBER 31, 1935

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 40,479.31		$ 40,479.31
Library Fund		7,471.45		7,471.45
Gifts		400.00		400.00
Instruction	$ 7,931.82	9,770.00		1,838.18
Research	4,145.52	12,470.00		8,324.48
Evening Lectures	83.99		$ 83.99	
Biological Bulletin and Membership Dues	7,790.04	8,805.35		1,015.31

Supply Department—Schedule VI	39,772.62	42,813.25		3,040.63
Mess—Schedule VII	21,611.81	21,349.57	262.24	
Dormitories—Schedule VIII	32,424.48	12,204.18	20,220.30	
(Interest and Depreciation charged to above three Departments—See Schedules VI, VII, and VIII)	35,260.33			35,260.33
Dividends, General Biological Supply House, Inc.		10,922.00		10,922.00
Rents:				
Danchakoff Cottages	352.63	750.00		397.37
Newman Cottage	88.80	250.00		161.20
Janitor's House	65.26	225.00		159.74
Railway and Garage		52.00		52.00
Sale of Duplicate Library Sets		58.05		58.05
Sundries		14.77		14.77
Maintenance of Plant:				
Buildings and Grounds	21,333.77		21,333.77	
Chemical and Special Apparatus	13,091.90		13,091.90	
Library Department Expense	7,598.02		7,598.02	
Truck Expense	849.94		849.94	
Sundry Expense	25.00		25.00	
Workmen's Compensation Insurance	553.16		553.16	
General Expenses:				
Administration Expenses	15,208.57		15,208.57	
Endowment Fund Trustee	968.50		968.50	
Bad Debts	568.21		568.21	
Reserve for Depreciation	43,625.42		43,625.42	
	$182,829.13	$168,034.93	$124,389.02	$109,594.82
Excess of Expenses over Income carried to Current Surplus—Exhibit C		14,794.20		14,794.20
		$182,829.13		$124,389.02

EXHIBIT C

Marine Biological Laboratory, Current Surplus Account, Year Ended December 31, 1935

Balance, January 1, 1935	$148,510.68
Add:	
Reserve for Depreciation charged to Plant Funds	43,625.42
	$192,136.10

Deduct:			
Payments from Current Funds during Year for Plant Assets as Shown in Schedule IV,			
Buildings		$ 2,012.64	
Equipment		5,229.08	
Library, Books, etc.		14,845.62	
		$22,087.34	
Less amount received for Plant Assets disposed of and charged to Current Account		690.00	
		$21,397.34	
Pensions and Allowances Paid	$4,560.00		
Less Income of Retirement Fund Received	861.82	3,698.18	
Excess of Expenses over Income for Year as shown in Exhibit B		14,794.20	39,889.72
Balance, December 31, 1935—Exhibit A			$152,246.38

Respectfully submitted,
LAWRASON RIGGS, JR.,
Treasurer

V. THE REPORT OF THE LIBRARIAN

We report for the year 1935 an increase in the Library budget of $500 specifically for the purchase of back sets, an activity practically at a standstill since 1933, when there was a cut of $5,000 from our budget. In the years 1933–34, the cost of serials being then at its maximum, the small amount that we had for back sets was used principally to retain all of our current serial titles intact. This year the expenditures were more normal in current serials and back sets and at the same time the binding was restored to normal. Library expenditures gave practically a balanced budget in each assignment apportioned as in former years: books, $300; serials, $6,000; binding, $1,500; express, $300; supplies, $500; back sets, $2,350; salaries, $7,150; total, $18,100; and applied as follows: books, $298.64; serials, $5,473.24; binding, $1,292.26; express, $97.96; supplies, $345.35; back sets, $3,496.08; salaries, $7,150.00; total, $18,153.53.

The scientific literature acquired by the Library in 1935 was therefore in great contrast to that of 1933–34, not only in back sets, 24 completed and 17 partially completed, as against 8 completed and 16 partially completed, but also in current serial titles which were increased by 74, total now received, 1,271: 359 purchased by the Marine Biological Laboratory (9 new), 37 by the Woods Hole Oceanographic Institution, 602 by exchange with the "Biological Bulletin" (21 new), 34 by exchange for the publications of the Oceanographic Institution (15 new), 225 as gifts to the former (25 new) and 14 to the latter (4 new). The Library acquired 158 new books, 93 by purchase and 41 by gift to the Marine Biological Laboratory, 21 purchased by the Woods Hole Oceanographic Institution and 3 gifts. Thirteen authors presented books and the other gifts were acquired through the courtesy of publishers.

The Library now contains 40,180 volumes and 91,641 reprints. Of the 4,478 reprints received this year, 927 were issued in serials of the year 1935. This figure is interesting because it is higher than at any time since we began in 1932 to make a separate check of the current year reprint receipts.

A new plan for current reprints will be begun this summer. They will be catalogued each day and placed for inspection on a special table in the reading room before they are filed away in the authors' boxes. We hope thus to make the new reprints more useful and at the same time to encourage our investigators to keep their files of reprints in the Library to date and complete.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I beg to submit herewith a report of the forty-eighth session of the Marine Biological Laboratory for the year 1935.

1. *Attendance.* Following the sharp decline in attendance in 1932 from the previous high figure of 362, the number of regularly registered investigators at the Laboratory has remained almost stationary, with only minor fluctuations from year to year. The exact figures for the past 5 years are: 362 in 1931, 314 in 1932, 319 in 1933, 323 in 1934 and 315 in 1935. The number of students in the courses has likewise remained practically constant at the limit set by the sizes of the available class-rooms. The total number of students and investigators together, after allowing for duplications, of 429 in 1935 represents approximately the optimum for the present facilities of the Laboratory, since it is just sufficient to fill all the available space comfortably and without undue crowding. Particularly noteworthy in 1935 was the large number of institutions represented by investigators. Both this number, which was 111, and the total of 143 for investigators and students combined, were the largest in the history of the Laboratory. The usual tabulation of the seasonal distribution of attendance at ten-day intervals for the past 9 years follows:

		1927	1928	1929	1930	1931	1932	1933	1934	1935
May	30	7	15	9	6	6	8	11	12	11
June	10	50	64	55	50	51	54	46	54	43
"	20	114	140	139	153	153	127	129	137	127
"	30	212	240	197	208	217	172	184	196	174
July	10	247	281	238	253	258	225	235	249	226
"	20	247	282	242	250	273	245	253	256	232
"	30	245	272	249	253	281	248	255	248	257
August	10	234	250	256	254	302	257	261	264	245
"	20	208	226	243	245	280	236	244	250	235
"	30	168	183	220	204	239	190	205	211	192
September	10	110	112	157	122	136	129	117	93	94
"	20	50	43	59	44	69	58	45	38	26
"	30	12	14	14	8	14	13	12	9	11

2. *The Report of the Treasurer.* In 1935, for the first time in four years, the annual decline in the income of the Laboratory from its endowment funds has been replaced by a slight increase. Though this increase amounted to slightly less than a thousand dollars, and though the total income from endowment of $47,950.76 is still nearly ten thousand dollars below that of pre-depression years, it is nevertheless very gratifying to be able to report an actual reversal of the previous trend. An inspection of Exhibit B from the Auditors' Report also reveals an increase of approximately $1,000.00 in the net income from the Supply Department (though the gross receipts were less than those for 1934), and similarly, for the first time in 4 years, there has been a slight but encouraging increase in the receipts from the rental of research space. Particularly helpful to the Laboratory at a time when its regular income was at nearly its lowest level since the establishment of its present endowment fund was a second special dividend from the General Biological Supply House, this dividend representing profits accumulated but not distributed during a period when business uncertainties made a relatively large reserve seem desirable.

Though in view of all the circumstances the present financial situation of the Laboratory is very satisfactory, it should nevertheless be noted that the annual gross income in 1935 was nearly forty thousand dollars less than that in 1931 and that throughout the period of the depression the budget has been kept balanced only by drastic economies of various sorts, some of which cannot much longer be continued without detriment to the scientific activities of the institution. It should likewise be remembered that for a considerable number of years to come the income of the Laboratory will be adversely affected by the gradual maturing of securities in its endowment fund, the proceeds from which must of necessity be reinvested at lower rates of interest. While the financial position of the Laboratory is therefore sound, and indeed extremely fortunate as compared with that of most other scientific and educational institutions, it is not at present such as to justify any departure from the very conservative policy with regard to expenditures that has been followed for the past four years.

3. *The Report of the Librarian.* Though it has not yet become possible to restore the earlier rate of growth of the library, temporarily checked by the reduction in the income from endowment funds, it is nevertheless encouraging to record very satisfactory gains during the past year. In particular, the increase in the number of journals currently received, amounting to 74 for the year in question, has been the largest for any single year since 1931. Substantial progress in the completion of back sets of journals has also been made. As the number of reprints in the library approaches the 100,000 mark, the attention of all members of the Corporation is invited to the desirability of transferring to this collection reprints in their own possession which are little or not at all used, but which might be of great value to other workers at the Laboratory. The growth of the Library since 1925 is concisely summarized in the following table:

	1925	1926	1927	1928	1929	1930	1931	1932	1933	1934	1935
Serials received currently	500	628	764	874	985	1,060	1,080	1,126	1,137	1,197	1,271
Total number of bound volumes..	15,000	18,200	22,800	26,500	28,300	31,500	33,800	36,000	37,400	38,600	40,200
Reprints	25,000	38,000	43,000	51,000	59,000	64,000	70,000	76,000	81,000	86,000	92,000

4. *Lectures and Scientific Meetings.* A complete list of the various lectures and scientific papers presented during the summer of 1935 will be found below (pages 28 to 32). The number of general lectures was 11, while there were also held 8 evening meetings and an all-day scientific session devoted to work accomplished at the Laboratory during 1935, at which 67 shorter papers were presented and discussed. Abstracts of most of these shorter papers, which cover a wide range of scientific activities, will be found in the Biological Bulletin for October, 1935.

5. *Apparatus Committee.* The great diversity in the character of the investigations carried on at the Marine Biological Laboratory and particularly the increasing complexity of the apparatus needed for their successful prosecution has for some time rendered desirable an advisory body, analogous to the Library Committee, the members of which can give advice concerning the purchase of special types of equipment of whose use they have expert knowledge, and who in cooperation with the Technical Manager can determine questions of general policy with regard to the most effective use of the valuable apparatus already in the

possession of the Laboratory. To meet this need, following authorization by the Executive Committee, President Lillie in the early summer of 1935 appointed a Committee of three consisting of Drs. Garrey, Harvey and Heilbrunn, which was later enlarged to the following membership: Drs. W. R. Amberson, D. J. Edwards, W. E. Garrey, E. N. Harvey, M. H. Jacobs and L. V. Heilbrunn, Chairman. During the summer of 1935 extremely useful work was accomplished by the original committee of three in making a general survey of all the apparatus belonging to the Laboratory and in securing from individual investigators a large number of valuable suggestions. At a meeting of the full committee held in September a series of general recommendations based on the information so obtained was drawn up. These recommendations should serve as a very sound basis for the future policies of the Laboratory in this important and highly technical field.

6. *Board of Trustees.* At the meeting of the Corporation held on Tuesday, August 13, 1935 the long and valuable services on the Board of Trustees of Professor G. H. Parker, whose membership began in 1908, and of Professor W. M. Wheeler, whose membership began in 1919, received recognition by the election of both to the permanent position of Trustee Emeritus. To fill the vacancies thus created the Corporation elected Professor C. E. McClung (Class of 1936) and Dr. A. H. Sturtevant (Class of 1939) respectively. Professor Laurence Irving was also chosen to succeed Professor W. C. Curtis as a member of the Class of 1939.

There are appended as parts of the report:

1. The Staff, 1935.
2. Investigators and Students, 1935.
3. A Tabular View of Attendance, 1931–1935.
4. Subscribing and Cooperating Institutions, 1935.
5. Evening Lectures, 1935.
6. Shorter Scientific Papers, 1935.
7. Members of the Corporation, August, 1935.

Respectfully submitted,

M. H. JACOBS,
Director.

1. THE STAFF, 1935

MERKEL H. JACOBS, *Director,* Professor of General Physiology, University of Pennsylvania.

Associate Director: ——

ZOÖLOGY

I. INVESTIGATION

GARY N. CALKINS, Professor of Protozoölogy, Columbia University.
E. G. CONKLIN, Professor of Zoölogy, Princeton University.
CASWELL GRAVE, Professor of Zoölogy, Washington University.
H. S. JENNINGS, Professor of Zoölogy, Johns Hopkins University.
FRANK R. LILLIE, Professor of Embryology Emeritus, The University of Chicago.
C. E. MCCLUNG, Professor of Zoölogy, University of Pennsylvania.
S. O. MAST, Professor of Zoölogy, Johns Hopkins University.
T. H. MORGAN, Director of the Biological Laboratory, California Institute of Technology.
G. H. PARKER, Professor of Zoölogy Emeritus, Harvard University.
E. B. WILSON, Professor of Zoölogy, Columbia University.
LORANDE L. WOODRUFF, Professor of Protozoölogy, Yale University.

II. INSTRUCTION

T. H. BISSONNETTE, Professor of Biology, Trinity College.
E. C. COLE, Professor of Biology, Williams College.
C. E. HADLEY, Associate Professor of Biology, New Jersey State Teachers College at Montclair.
F. R. KILLE, Instructor in Zoölogy, Swarthmore College.
S. A. MATTHEWS, Associate in Anatomy, School of Medicine, University of Pennsylvania.
O. E. NELSEN, Instructor in Zoölogy, University of Pennsylvania.
L. P. SAYLES, Assistant Professor of Biology, College of the City of New York.

JUNIOR INSTRUCTORS

A. J. WATERMAN, Assistant Professor of Biology, Williams College.
F. H. WOODS, Assistant Professor of Zoölogy, University of Missouri.

PROTOZOÖLOGY

I. INVESTIGATION

(*See Zoölogy*)

II. INSTRUCTION

GARY N. CALKINS, Professor of Protozoölogy, Columbia University.
ELIZABETH DRUMTRA, Instructor in Zoölogy, Wilson College.
G. W. KIDDER, Instructor in Zoölogy, College of the City of New York.

EMBRYOLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

L. G. Barth, Instructor of Experimental Zoölogy, Columbia University.
Hubert B. Goodrich, Professor of Biology, Wesleyan University.
Benjamin H. Grave, Professor of Biology, De Pauw University.
Leigh Hoadley, Professor of Zoölogy, Harvard University. (Absent in 1935.)
Charles Packard, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.
Oscar Schotté, Assistant Professor of Biology, Amherst College.

PHYSIOLOGY

I. Investigation

William R. Amberson, Professor of Physiology, University of Tennessee.
Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.
Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.
Ralph S. Lillie, Professor of General Physiology, The University of Chicago.
Albert P. Mathews, Professor of Biochemistry, University of Cincinnati.

II. Instruction

Teaching Staff

Robert Chambers, Professor of Biology, New York University.
J. K. W. Ferguson, Assistant Professor of Physiology, University of Western Ontario.
Rudolf Höber, Visiting Professor of Physiology, University of Pennsylvania.
Laurence Irving, Professor of Experimental Biology, University of Toronto.
Leonor Michaelis, Member of the Rockefeller Institute, New York City.
C. Ladd Prosser, Assistant Professor of Physiology, Clark University.

Junior Instructors

Kenneth Fisher, Demonstrator in Biology, University of Toronto.
F. J. M. Sichel, Instructor in Zoölogy, University of Pennsylvania.

BOTANY

I. Investigation

C. E. Allen, Professor of Botany, University of Wisconsin.
S. C. Brooks, Professor of Zoölogy, University of California.
B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.
Ivey F. Lewis, Professor of Biology, University of Virginia.
Wm. J. Robbins, Professor of Botany, University of Missouri.

II. Instruction

William Randolph Taylor, Professor of Botany, University of Michigan.
G. W. Prescott, Assistant Professor of Biology, Albion College.

GENERAL OFFICE

F. M. MacNaught, Business Manager.
Polly L. Crowell, Assistant.
Edith Billings, Secretary.

RESEARCH SERVICE AND GENERAL MAINTENANCE

Samuel E. Pond, Technical Manager.
Oscar W. Richards, Chemical Service.
G. Failla, X-Ray Physicist.
Thomas E. Larkin, Superintendent.
William Hemenway, Carpenter.
Lester F. Boss, Research Technician.
J. D. Graham, Glassblower.
P. H. Liljestrand, Assistant.

LIBRARY

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.), Librarian.
Deborah Lawrence, Secretary.
Doris Endrejat, Mary A. Rohan, Assistants.

SUPPLY DEPARTMENT

James McInnis, Manager.
Milton B. Gray, Collector.
A. M. Hilton, Collector.
A. W. Leathers, Shipping Department.
Geoffrey Lehy, Collector.
Walter Kahler, Collector.
Ruth S. Crowell, Secretary.
Anna N. Hall, Secretary.

MUSEUM

George M. Gray, Curator Emeritus.

2. INVESTIGATORS AND STUDENTS, 1935

Independent Investigators

Abramowitz, Alexander A., Assistant in Biology, Harvard University.
Adams, James A., Fellow, Iowa State College.
Adolph, E. F., Associate Professor of Physiology, University of Rochester, School of Medicine.
Albaum, Harry G., Graduate Student, Columbia University.
Alexander, Gordon, Associate Professor of Biology, University of Colorado.
Alvey, Clifford H., Instructor in Parasitology, Purdue University.
Amberson, William R., Professor of Physiology, College of Medicine, University of Tennessee.
Anderson, R. L., Professor of Biology, Johnson C. Smith University.
Appel, F. W., Associate Professor of Biology, St. John's College.
Baker, Clinton L., Professor of Biology, Southwestern.

Ball, Eric G., Associate, Department of Physiological Chemistry, Johns Hopkins Medical School.
Barth, L. G., Instructor in Embryology, Columbia University.
Bissonnette, T. Hume, Professor and Head of Biology Department, Trinity College.
Bostian, C. H., Assistant Professor of Zoölogy, North Carolina State College.
Bowen, R. E., Assistant Professor of Biology, Long Island University.
Boyd, James D., Rockefeller Fellow, Queens University, Belfast, Ireland.
Bozler, Emil, Fellow in Medical Physics, Johnson Foundation, University of Pennsylvania.
Bradley, H. C., Professor of Physiological Chemistry, University of Wisconsin.
Brinley, Floyd J., Assistant Professor of Zoölogy, North Dakota State College.
Brown, Dugald E. S., Assistant Professor of Physiology, New York University.
Budington, R. A., Professor of Zoölogy, Oberlin College.
Burton, A. C., General Education Training Fellowship, University of Pennsylvania.
Cable, Raymond M., Associate Professor of Biology, Berea College.
Calkins, Gary N., Professor of Protozoölogy, Columbia University.
Carlson, J. Gordon, Instructor in Biology, Bryn Mawr College.
Carpenter, Esther, Instructor in Zoölogy, Smith College.
Carpenter, Russell L., Assistant Professor of Anatomy, College of Physicians and Surgeons, Columbia University.
Cattell, Ware, Associate Editor, Scientific Monthly.
Chambers, Robert, Research Professor of Biology, Washington Square College, New York University.
Cheney, Ralph H., Professor of Biology, Long Island University.
Clark, Eleanor Linton, University of Pennsylvania, Medical School.
Clark, Eliot R., Professor of Anatomy, University of Pennsylvania, Medical School.
Clark, Leonard B., Assistant Professor of Biology, Union College.
Clowes, G. H. A., Director of Research, Lilly Research Laboratories.
Coe, W. R., Professor of Biology, Yale University.
Cole, Elbert C., Professor of Biology, and Chairman of Department, Williams College.
Conklin, Edwin G., Professor Emeritus of Biology, Princeton University.
Coonfield, B. R., Assistant Professor, Brooklyn College.
Copeland, Manton, Professor of Biology, Bowdoin College.
Costello, Donald P., National Research Fellow in Zoölogy, Hopkins Marine Station.
Cotui, Frank W., Associate Professor, Research Surgery, New York University, College of Medicine.
Cowles, R. P., Professor of Zoölogy, Johns Hopkins University.
Croasdale, Hannah T., Graduate Student, University of Pennsylvania.
Crowell, Prince S., Jr., Instructor, Harvard University.
Danforth, Louise, Graduate Alumna, American University.
Davis, J. E., Research Assistant, The University of Chicago.
Dieter, Clarence D., Acting Head of Biology, Washington and Jefferson College.
Diller, William F., Instructor in Zoölogy, Dartmouth College.
Donaldson, Henry H., Member, Wistar Institute.
Doyle, William L., General Education Board Fellow, The Johns Hopkins University.
Dreyer, W. A., Instructor in Zoölogy, University of Cincinnati.
Drumtra, Elizabeth, Assistant in Zoölogy, Barnard College, Columbia.
DuBois, Eugene F., Professor of Medicine, Cornell University Medical College.
duBuy, Herman G., Fellow, Harvard Medical School.

Edwards, Dayton J., Associate Professor of Physiology, Cornell University Medical College.

Fenn, Wallace O., Professor of Physiology, University of Rochester, School of Medicine and Dentistry.

Ferguson, J. K. W., Lecturer in Physiology, University of Western Ontario Medical School.

Fischer, Ernst, Visiting Associate in Physiology, Medical School, University of Rochester.

Fleisher, Moyer S., Professor of Bacteriology and Hygiene, St. Louis University.

Fry, Henry J., Visiting Investigator, Cornell University Medical College.

Fuchs, William B., American University.

Garrey, W. E., Professor of Physiology, Medical School of Vanderbilt University.

Gojdics, Mary, Assistant Professor of Biology, Duchesne College.

Goodrich, H. B., Professor of Biology, Wesleyan University.

Gottschall, Gertrude G., Assistant in Biochemistry, Cornell University Medical College.

Grave, B. H., Director, Department of Zoölogy, DePauw University.

Grave, Caswell, Professor of Zoölogy, Washington University.

Guest, George M., Associate Professor of Pediatrics, University of Cincinnati Medical College.

Hadley, Charles E., Associate Professor of Biology, New Jersey State Teachers' College at Montclair.

Hague, Florence, Assistant Professor of Zoölogy, Sweet Briar College.

Hanström, Bertil, Professor of Zoölogy, Director of the Zoological Institute, University of Lund, Sweden.

Harkins, Henry H., Research Chemist, U. S. Rubber Company.

Harnley, Morris H., Assistant Professor, Washington Square College, New York University.

Harvey, Ethel Browne, Princeton University.

Hasler, Arthur D., Graduate Assistant, University of Wisconsin.

Haywood, Charlotte, Associate Professor of Physiology, Mount Holyoke College.

Heilbrunn, L. V., Associate Professor of Zoölogy, University of Pennsylvania.

Hendee, Esther C., Research Associate, University of California.

Henshaw, Paul S., Biophysicist, Memorial Hospital.

Hess, Walter N., Professor of Biology and Head of Department, Hamilton College.

Hibbard, Hope, Assistant Professor of Zoölogy, Oberlin College.

Hill, Samuel E., Assistant in General Physiology, Rockefeller Institute.

Höber, Rudolf, Visiting Professor of Physiology, University of Pennsylvania.

Hodge, Charles, 4th, Instructor, Temple University.

Holter, Heinz, Research Worker, Carlsberg Laboratory, Copenhagen.

Hook, Sabra J., Assistant Professor of Zoölogy, University of Rochester.

Howe, H. E., Editor, Industrial and Engineering Chemistry.

Huggins, John R., Graduate Student, University of Pennsylvania.

Hunt, Reid, Professor of Pharmacology, Harvard Medical School.

Hursh, John B., Fellow, Rochester Medical School.

Hyman, Libbie H., American Museum of Natural History.

Ives, Philip T., California Institute of Technology.

Irving, Laurence, Professor of Experimental Biology, University of Toronto.

Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.

Jenkins, George B., Professor of Anatomy, George Washington University.

Johlin, J. M., Associate Professor of Biochemistry, Vanderbilt University School of Medicine.

Jones, E. Elizabeth, Instructor in Zoölogy, Wellesley College.

Jones, Ruth McClung, Instructor in Zoölogy and Botany, Swarthmore College.
Kaufmann, Berwind P., Professor of Botany, University of Alabama.
Keil, Elsa M., Assistant Professor of Zoölogy, New Jersey College for Women.
Kidder, George W., Instructor, College of the City of New York.
Kille, Frank R., Instructor in Zoölogy, Swarthmore College.
Kilpatrick, Martin, Assistant Professor of Chemistry, University of Pennsylvania.
Kilpatrick, Mary L., University of Pennsylvania.
Kindred, James E., Associate Professor of Histology and Embryology, University of Virginia Medical School.
Kleinholz, L. H., Austin Fellow, Harvard University.
Knower, Henry McE., Research Associate in Biology, Yale University.
Knowlton, Frank P., Professor of Physiology, College of Medicine, Syracuse University.
Kreezer, George, Research Associate, Training School at Vineland, New Jersey.
Kuyper, Adrian C., Instructor, State University of Iowa.
Lancefield, Donald E., Associate Professor of Zoölogy, Columbia University.
Lancefield, Rebecca C., Associate in Bacteriology, Rockefeller Institute.
Laug, Edwin P., Assistant in Physiology, University of Pennsylvania.
Lillie, Prof. Frank R., Professor of Embryology, Emeritus, The University of Chicago.
Lillie, Prof. Ralph S., Professor of General Physiology, The University of Chicago.
Long, Margaret E., Graduate Student, University of Pennsylvania.
Lucas, Alfred M., Assistant Professor of Zoölogy, University of Iowa.
Lucas, Miriam Scott, Instructor in Cytology, Washington University School of Medicine.
Magruder, Samuel R., Laboratory Instructor, University of Cincinnati.
Manwell, Reginald D., Associate Professor of Zoölogy, Syracuse University.
Marsland, Douglas A., Assistant Professor of Biology, Washington Square College, New York University.
Martin, Earl A., Chairman of Biology Department, Brooklyn College.
Marquette, William G., Graduate Student, Columbia University.
Mast, S. O., Professor of Zoölogy, The Johns Hopkins University.
Mathews, Albert P., Professor of Biochemistry, University of Cincinnati.
Matthews, Samuel A., Associate Professor of Anatomy, University of Pennsylvania.
McClung, C. E., Director, Department of Zoölogy, University of Pennsylvania.
Michaelis, Leonor, Member, Rockefeller Institute for Medical Research.
Monné, Ludwik, Rockefeller Fellow, University of Lwow, Poland.
Morgan, Lilian V., Pasadena, California.
Morgan, T. H., Professor of Experimental Zoölogy, California Institute of Technology.
Morill, Charles V., Associate Professor of Anatomy, Cornell University Medical College.
Muratori, Giulio, Rockefeller Fellow, Institute of Anatomy, R. University of Padova, Italy.
Navez, Albert E., Lecturer in Physiology, Harvard University.
Nelsen, Olin E., Assistant Professor of Zoölogy, University of Pennsylvania.
Nonidez, José F., Assistant Professor of Anatomy, Cornell University Medical College.
Northrup, John H., Member, Rockefeller Institute.
Novikoff, Alex B., Tutor, Brooklyn College.
Orr, Paul R., Instructor, Brooklyn College.
Osterhout, Winthrop J. V., Member, Rockefeller Institute for Medical Research.

Packard, Charles, Assistant Professor, Institute of Cancer Research, Columbia University.
Parker, G. H., Professor of Zoölogy, Emeritus, Harvard University.
Parpart, Arthur K., Assistant Professor of Physiology, Princeton University.
Patrick, Ruth, Temple University.
Plough, Harold H., Professor of Biology, Amherst College.
Pond, Samuel E., Technical Manager, Marine Biological Laboratory.
Prescott, Gerald W., Assistant Professor of Biology, Albion College.
Prosser, C. Ladd, Assistant Professor of Physiology, Clark University.
Puckett, William O., Instructor in Biology, Princeton University.
Pumphrey, Richard J., Rockefeller Fellow, Johnson Foundation, University of Pennsylvania.
Ramsey, Robert W., Instructor in Zoölogy, University of Rochester.
Rice, Kenneth S., Woods Hole, Massachusetts.
Richards, Oscar W., Instructor in Biology, Yale University.
Robertson, Charles W., Assistant Instructor, Washington Square College, New York University.
Root, Walter S., Associate Professor of Physiology, Syracuse Medical School.
Rugh, Roberts, Instructor in Zoölogy, Hunter College.
Sampson, Myra M., Professor of Biology and Head of Department, Smith College.
Sandow, Alexander, Instructor in Biology, Washington Square College, New York University.
Saslow, George, Assistant Professor of Biology, Washington Square College, New York University.
Sayles, Leonard P., Assistant Professor of Biology, College of the City of New York.
Schmidt, Ida Genther, Instructor in Anatomy, College of Medicine, University of Cincinnati.
Schmidt, L. H., Research Fellow in Biochemistry, Christ Hospital and College of Medicine, University of Cincinnati.
Schotté, Oscar E., Assistant Professor of Biology, Amherst College.
Schrader, Franz, Professor of Zoölogy, Columbia University.
Schrader, Sally Hughes, Professor of Zoölogy, Sarah Lawrence College.
Scott, Allan C., Assistant in Zoölogy, Columbia University.
Shapiro, Herbert, Research Council Fellow in the Biological Sciences, The University of Chicago.
Shaw, Myrtle, Senior Bacteriologist, Division of Laboratories and Research, New York State Department of Health.
Shoup, Charles S., Assistant Professor of Biology, Vanderbilt University.
Sichel, Ferdinand J. M., Instructor, University of Pennsylvania.
Slifer, Eleanor H., Associate in Zoölogy, University of Iowa.
Smith, Carl G., Demonstrator in Physiology, University of Toronto.
Smith, Dietrich C., Instructor in Physiology, College of Medicine, University of Tennessee.
Solberg, Archie N., Assistant in Zoölogy, Columbia University.
Speicher, Kathryn G., Pennsylvania College for Women.
Speidel, Carl C., Professor of Anatomy, University of Virginia Medical School.
Steinbach, Henry B., Instructor in Zoölogy, University of Rochester Medical School.
Stern, Curt, Assistant Professor, Department of Zoölogy, University of Rochester.
Stewart, Dorothy R., Assistant Professor of Biology, Skidmore College.
Stiehler, Robert D., Research Fellow, Johns Hopkins University.
Stockard, Charles R., Professor of Anatomy, Cornell Medical College.

Strong, Oliver S., Professor of Neurology and Neuro-Histology, College of Physicians and Surgeons, Columbia University.
Stunkard, H. W., Professor of Biology, New York University.
Sturtevant, A. H., Professor of Genetics, California Institute of Technology.
Summers, Francis M., Instructor in Biology, Bard College, Columbia University.
Sumwalt, Margaret, Johns Hopkins University Medical School.
Tashiro, Shiro, Professor of Biochemistry, University of Cincinnati.
Taylor, Wm. Randolph, Professor of Botany, University of Michigan.
Teorell, E. Torsten, Docent in Physiological Chemistry, The Karolinska Institutet.
TeWinkel, Lois E., Assistant Professor of Zoölogy, Smith College.
Trager, William, Assistant, Rockefeller Institute.
Vicari, Emilia, Research Associate in Anatomy, Cornell University Medical College.
Waterman, Allyn J., Assistant Professor of Biology, Williams College.
Weiss, Paul, Assistant Professor of Zoölogy, The University of Chicago.
Whedon, Arthur D., Professor of Zoölogy and Physiology and Head of Department, North Dakota Agricultural College.
Whiting, Anna R., Professor, Head of Department of Biology, Pennsylvania College for Women.
Whiting, P. W., Visiting Investigator, Carnegie Institution of Washington, Cold Spring Harbor.
Wilbrandt, Walter, Rockefeller Fellow, University of Pennsylvania.
Willier, B. H., Professor of Zoölogy and Head of Department, University of Rochester.
Wilson, Edmund B., Da Costa Professor Emeritus in Residence, Columbia University.
Wintrobe, Maxwell M., Associate in Medicine, Johns Hopkins University.
Wodehouse, R. P., Director of the Protein Laboratory, The Arlington Chemical Company.
Wolf, E. Alfred, Assistant Professor of Zoölogy, University of Pittsburgh.
Woodruff, Lorande L., Professor of Protozoölogy, Yale University.
Woods, Farris H., Assistant Professor of Zoölogy, University of Missouri.
Woodward, Alvalyn E., Assistant Professor of Zoölogy, University of Michigan.
Young, Roger A., Assistant Professor, Howard University.
Zirkle, Conway, Associate Professor, University of Pennsylvania.

Beginning Investigators

Angerer, C. A., Harrison Fellow, University of Pennsylvania.
Baker, Stanley, Instructor in Zoölogy, Wabash College.
Bosworth, Millard W., Dennison Research Assistant, Wesleyan University.
Bullowa, Elizabeth, Student, College of Physicians and Surgeons, Columbia University.
Churney, Leon, Instructor in Zoölogy, University of Pennsylvania.
Clark, Jean M., Graduate Student, University of Pennsylvania.
Cohen, A. A., Harvard University.
Copeland, D. Eugene, Rochester University.
DeBoer, Benjamin, Graduate Assistant, University of Missouri.
Denny, Martha, Student, Radcliffe College.
Derrickson, Mary B., Assistant, Vassar College.
Donnellon, James A., University of Pennsylvania.
Dordick, Isadore, University of Pennsylvania.
Farrow, John, Graduate Student, University of Pennsylvania.
Friedman, Sam, Washington Square College, New York University.
Guttman, Rita M., Graduate Student, Columbia University.

Guttman, Samuel A., Assistant Instructor, Department of Physiology, Cornell University Medical College.
Haar, Franklin B., Graduate Student, University of Pittsburgh.
Hall, John F., Assistant in Physiology, Princeton University.
Hawley, Katharine J., Student, Smith College.
Hollingsworth, Josephine, Graduate Student, University of Pennsylvania.
Hoover, Earl, Johns Hopkins University.
Hornor, Helen B., Teaching Assistant, Barnard College, Columbia University.
Hunter, Francis, Part-time Assistant and Graduate Student, Princeton University.
Hunter, Laura N., Graduate Student, University of Pennsylvania.
Itoh, Hidegoroh, Graduate Student, University of Pennsylvania.
Jailer, Joseph W., Graduate Student, Columbia University.
Jakobsen, Edith M., New Jersey State Teachers' College.
Kaliss, Nathan, Assistant in Zoölogy, Columbia University.
Keltch, Anna K., Research Chemist, Lilly Research Laboratories.
Kintner, Kenneth, Assistant, Purdue University.
Koonz, Carl H., Northwestern University.
Krahl, Maurice E., Research Physical Chemist, Lilly Research Laboratories.
Leovey, Francis, Assistant in Department of Physiology, University of Budapest, Budapest, Hungary.
Lippman, Richard W., Student, Yale University.
McBride, T. F., Instructor in Clinical Dentistry, University of Pittsburgh.
McKinniss, Mary, Student, University of Pittsburgh.
Mazia, Daniel, Instructor in Zoölogy, University of Pennsylvania.
Moser, Floyd, Graduate Student, University of Pennsylvania.
Ormsby, Louise, Graduate Student, Columbia University.
Palmer, Louise, Instructor, Wellesley College.
Peabody, Elizabeth B., Radcliffe College.
Piercy, Robert Lee, Student, School of Medicine, University of Rochester.
Podolnick, Nelson, University of Pennsylvania.
Reeder, Elizabeth M., Instructor in Zoölogy, University of Missouri.
Reinhard, Edward G., Associate Professor of Biology, St. Thomas College.
Ritchie, Laurence, Part-time Instructor, Northwestern University.
Spofford, Walter R., Graduate Student, Yale University.
Stanbruy, John, Duke University.
Tyson, Rebecca J., Wayne University.
Valenstein, Arthur F., Medical Student, Cornell University Medical College.
Webster, Edward C., Teaching Assistant, New York University.
Wentsler, Norman E., Student, University of Pennsylvania.
Wichterman, Ralph, Graduate Student, University of Pennsylvania.
Woodward, H. E., Research Assistant, University of Toronto.
Zuck, Robert K., Oberlin College.

Research Assistants

Atchley, Dana W., Jr., Technician, Rockefeller Institute.
Beck, Lyle V., Research Assistant, University of Pennsylvania.
Bishop, Daniel W., Swarthmore College.
Brambel, Charles E., Instructor in Zoölogy, Johns Hopkins University.
Brandwein, Paul F., Assistant, New York University.
Brower, Helen Porter, Laboratory Assistant, Harvard University.
Carlson, Sven, University of Lund.
Clark, John K., Undergraduate Assistant, Trinity College.
Corey, H. Irene, Research Assistant, University of Pennsylvania.
Deehan, Sylvester J., II, University of Pennsylvania.

Fennell, Richard A., Assistant, Johns Hopkins University.
Fisher, Kenneth C., Fellow in the Department of Physiology, University of Toronto.
Flynn, Carl M., Instructor in Zoölogy, University of Maine.
Glassman, Harold N., Graduate Student, University of Pennsylvania.
Godrich, Jack, Photographer, Columbia University.
Goffin, Catherine E., Research Assistant, Lilly Research Laboratories.
Goldin, Abraham, Laboratory Assistant, Brooklyn College.
Grand, C. G., Research Associate, Washington Square College, New York University.
Hershkowitz, Solomon G., Columbia University.
Hibbard, Jeanne, 245 W. College St., Oberlin, Ohio.
Hill, Edgar S., Research Assistant, Washington University Medical School.
Höber, Josephine, University of Pennsylvania Medical School.
Holmes, Dorothy B., Assistant Bacteriologist, New York State Department of Health.
Johnson, James B., Research Assistant, DePauw University.
Kershaw, Margaret A., Research Assistant, Wheaton College.
Koppleman, Samuel, University of Pennsylvania.
Kraatz, Charles, Graduate Assistant in Zoology, University of Cincinnati.
Lehman, Eleanor M., Assistant, University of Pittsburgh.
Pappenheimer, John R., Student, Harvard University.
Petrillo, Vincent A., Technician, Rockefeller Institute.
Pinson, Ernest A., Assistant, DePauw University.
Rawles, Mary E., Research Assistant, University of Rochester.
Richardson, Margaret, Technician, Columbia University.
Robertson, Kathleen M., Research Fellow, University of Toronto.
Robertson, Lola E., Research Assistant, New York University.
Rogers, Lotta M., Instructor in Biology, Albion College.
Rose, Sylvan M., Graduate Assistant, Amherst College.
Sanders, Elmer K., Student, Medical School, Vanderbilt University.
Sasaki, Yasuo, Graduate Student, University of Cincinnati.
Shapiro, Arthur, 192 Fifth Avenue, Brooklyn, New York.
Shaw, Isidor, Technical Assistant in Biology, Long Island University.
Smith, Jay A., Laboratory Assistant, DePauw University.
Smythe, C. V., Assistant in Physical Chemistry, Rockefeller Institute.
Speicher, B. R., Research Assistant, Amherst College.
Stix, Helen D., Teaching Assistant, University of Cincinnati.
Taylor, John F., Graduate Student, Johns Hopkins University.
Thornton, Charles S., Assistant in Biology, Princeton University.
Warren, Marshall R., Research Assistant, University of Cincinnati.
Williams, William E., Student, Williams College.
Wohnus, J. Fred, Assistant Instructor, Williams College.
Young, Saul B., Technician, Rockefeller Institute for Medical Research.

Students

BOTANY

Bazoll, Ida B., Assistant Intermediate, Hyde School, Boston, Massachusets.
Hawkins, Thelma E., Assistant Professor of Biology, Lincoln University.
Howes, Samuel A., Instructor in Biology and Chemistry, Groton School, Groton, Massachusetts.
Pease, Eleanor F., Wellesley College.
Stone, Winona E., Instructor in Botany, University of Vermont.
Zuck, Robert K., Oberlin College.

EMBRYOLOGY

Aplington, Henry W., Student, Wesleyan University.
Becket, Ronald S., Student, Amherst College.
Bridgman, Jane, Smith College.
Brill, Edmund R., Undergraduate, Harvard University.
Copeland, Donald E., University of Rochester.
Dalton, Howard C., Undergraduate Assistant, Wesleyan University.
Dansereau, Antonio, Professor of Biology, Montreal University.
Engel, Jean, Pennsylvania College for Women.
Frank, Rhoda D., Hunter College.
Frothingham, Margaret, Sarah Lawrence College.
Gnanadikan, Gnanambal, Graduate Student, Radcliffe College.
Gravett, Howard L., Assistant in Zoölogy Department, University of Illinois.
Hadley, Ruth G., Biology Teacher, Jenkintown, Pennsylvania.
Johnson, James B., Student Assistant, DePauw University.
Jones, E. Elizabeth, Instructor in Zoölogy, Wellesley College.
Karolyi, Elmer J., Assistant, Western Reserve University.
Keister, Margaret L., Student Assistant, Wheaton College.
Lillie, Emily Ann, The University of Chicago.
Mapp, F. Everett, Teacher, Atlanta University.
Mowry, Helen A., Associate Professor of Biology, Skidmore College.
Nunnemacher, Rudolph F., Graduate Student, Harvard University.
Potsubay, Samuel F., Jr., Undergraduate Student, Amherst College.
Radi, Mohamed H., Member, Egyptian Educational Mission.
Reeder, Elizabeth M., Instructor, University of Missouri.
Reinhard, Edward G., Associate Professor of Biology, St. Thomas College.
Schultz, Helen H., Assistant Professor of Biology, State Teachers College, Fredericksburg, Virginia.
Shaw, Roy C., Graduate Assistant, University of Rochester.
Smith, Jay Alfred, Laboratory Assistant, DePauw University.
Stewart, Walter A., Undergraduate Assistantship, Dartmouth College.
Taylor, Sarah P., Sarah Lawrence College.
Todd, Robert E., Austin Teaching Fellow, Harvard University.
Wall, Lester A., Jr., Laboratory Assistant, St. John's College.
Zahl, Paul A., Teaching Fellow, Harvard University.

PHYSIOLOGY

Bullowa, Elizabeth, Student, College of Physicians and Surgeons, Columbia University.
Burton, Alan C., General Education Board Training Fellowship, University of Pennsylvania.
DeBoer, Benjamin, Graduate Assistant, University of Missouri.
Dickerson, Virginia C., Mount Holyoke College.
Earl, Ruth R., Laboratory Assistant, Brooklyn College.
Evans, Eleanor M., University of Toronto.
Granick, Sam., Research Fellow in Plant Physiology, University of Michigan.
Greey, Elizabeth L., University of Toronto.
Hathaway, Charles O., Jr., Graduate Student, Laboratory Instructor, University of Virginia.
Hotchkiss, Rollin D., Fellow, Rockefeller Institute for Medical Research.
Kilburn, Virginia Thomas, Wellesley College.
Knight, Betty L., Graduate Student, Cornell University Medical College.
Lieneman, Catharine, Assistant Professor, Woman's College, University of North Carolina.
Lippman, Richard W., Student, Yale University.

Pappenheimer, John R., Student, Harvard University.
Robertson, Kathleen M., Research Fellow, University of Toronto.
Surrarrer, Thomas C., Assistant Professor, Baldwin-Wallace College.
Valenstein, Arthur F., Student, Cornell University Medical College.
Wagner, Paul R., Instructor in Biology, Ursinus College.
Woodward, Hubert E., Research Assistant, University of Toronto.

PROTOZOÖLOGY

Albaum, Harry G., Student, Columbia University.
Balamuth, William, Student, College of the City of New York.
Carpenter, Philip L., Clark University.
Crowen, Edwina L., Columbia University.
Hughes, Roscoe D., Assistant in Zoölogy, Columbia University.
Fronczak, Michael I., Seton Hall College.
Jailer, Joseph W., Graduate Student, Columbia University.
Kadushin, Miriam, Hunter College.
Koonz, Carl H., Graduate Assistant, Northwestern University.
Lilly, Daniel McQ., Instructor in Biology, Providence College.
Mirsky, Rachel, 1258 East 102 Street, Cleveland, Ohio.
Morholt, Evelyn, Columbia University.
Orange, Jeanette, Student, Columbia University.
Ritchie, Lawrence, Part-time Instructor, Northwestern University.
Rogers, Lotta M., Instructor in Biology, Albion College.
Schofer, Anna, Goucher College.

INVERTEBRATE ZOÖLOGY

Babcock, Virginia F., Head of Biology Department, Bronxville High School.
Bauer, C. Adele, Goucher College.
Baumgartner, Barbara, Butler University.
Bergren, Leslie, Instructor, University High School, Minneapolis.
Berkowitz, Philip, Teaching Fellow, Washington Square College, New York University.
Bledsoe, John A., Graduate Student, University of Cincinnati.
Bright, William M., Graduate Student, University of Illinois.
Brown, Relis B., Graduate Student, Yale University.
Cairns, John MacKay, Jr., Hamilton College.
Castle, Ruth M., New Jersey State Teachers' College at Montclair.
Chambers, William N., Student, Amherst College.
Child, Esther W., Bennington College.
Clark, Beatrice, Wellesley College.
Conant, Betsy D., Student, University of Rochester.
Conder, Evert, Research Assistant, University of Illinois.
Deyrup, Natalie J., Student, College of Physicians and Surgeons, Columbia University.
Doughty, Gertrude R., Student, Bennington College.
Dugal, Louis-Paul, Professeur au Collège Ste.-Marie.
English, James E., Student, University of Missouri.
Ferguson, Malcolm S., Assistant in Zoölogy, University of Illinois.
Galambos, Robert, Oberlin College.
Goodale, Marion P., Teacher, Middlebury College.
Gordon, Hazel E., University of Wisconsin.
Hansen, Donald F., Zoölogist, State Natural History Survey, University of Illinois.
Harpster, Hilda, Instructor, Sweet Briar College.
Hathaway, Charles O., Jr., Graduate Student, Laboratory Instructor, University of Virginia.
Hayes, Elizabeth A., Laboratory Assistant, Rockefeller Institute.

Henning, Willard L., Graduate Assistant, University of Missouri.
Henson, Margaret, Smith College.
Hewitt, Cornelia B., Smith College.
Hoyt, Dorothy, Student, Swarthmore College.
Hummel, Elizabeth S., New Jersey College.
Hutchens, John O., Undergraduate Assistant, Butler University.
Jakus, Marie A., Oberlin College.
Litwiller, Raymond W., The University of Chicago.
Martin, Walter E., Instructor in Zoölogy, Purdue University.
Mattox, Norman T., Graduate Assistant in Zoölogy, University of Illinois.
Mayo, Virginia, Graduate Assistant, Mount Holyoke College.
McConnell, Elma, New Jersey College.
Miller, Blanche, Instructor, Agnes Scott College.
Moseley, Russell L., Graduate Assistant, Wabash College.
Pease, Gwynneth, Laboratory Assistant, Wellesley College.
Renshaw, Birdsey, Assistant, Harvard University.
Robinson, Robert A., Harvard University.
Schmeichel, Norman L., Graduate Teaching Assistant, University of Wisconsin.
Schroeder, Nancy S., Sarah Lawrence College.
Shepard, Charles C., Wesleyan University.
Shettles, Landrum B., Graduate Student, Johns Hopkins University.
Snyder, Ruth E., Laboratory Assistant, Barnard College.
Stauffer, Robert C., Instructor in Biology, Dartmouth College.
Welsh, Wilfred R., Undergraduate Student, New Jersey State Teachers' College at Montclair.
Wilde, Walter S., Teaching Assistant, University of Minnesota.
Wilson, John Woodrow, Duke University.
Winternitz, Jane K., Vassar College.
Wistar, Raquilita, Wilson College.

3. TABULAR VIEW OF ATTENDANCE

	1931	1932	1933	1934	1935
Investigators—Total	362	314	319	323	315
Independent	236	212	210	222	208
Under Instruction	83	73	66	49	56
Research Assistants	43	29	43	52	51
Students—Total	125	132	118	131	130
Zoölogy	55	55	54	54	55
Protozoölogy	17	16	11	11	16
Embryology	29	29	28	30	33
Physiology	17	18	19	23	20
Botany	7	14	6	13	6
Total Attendance	487	446	437	454	445
Less Persons registered as both students and investigators	20	14	12	15	16
	467	432	425	439	429
Institutions Represented—Total	137	141	120	131	143
By Investigators	102	94	92	98	111
By Students	68	76	58	75	70
Schools and Academies Represented					
By Investigators	–	–	1	1	–
By Students	4	1	2	5	3
Foreign Institutions Represented					
By Investigators	8	8	5	4	7
By Students	1	1	–	1	1

4. SUBSCRIBING AND COÖPERATING INSTITUTIONS IN 1935

Amherst College
Atlanta University
Barnard College
Berea College
Bowdoin College
Bryn Mawr College
Butler College
California Institute of Technology
Children's Hospital, Cincinnati, Ohio
Christ Hospital, Cincinnati, Ohio
College of Physicians & Surgeons
Columbia University
Cornell University
Cornell University Medical College
DePauw University
Duke University
General Education Board
Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Hunter College
Industrial & Engineering Chemistry, of the American Chemical Society
Iowa State College
Johns Hopkins University
Johnson C. Smith University
Eli Lilly & Co.
Lincoln University
Long Island University
Memorial Hospital, New York City
Morehouse College
Mount Holyoke College
New York State Department of Health
New York University
New York University Medical School
Northwestern University
Oberlin College
Pennsylvania College for Women
Princeton University
Purdue University
Radcliffe College
Rockefeller Foundation
Rockefeller Institute for Medical Research
Royal Egyptian Legation
St. John's College
Seton Hall College
Smith College
Swarthmore College
Syracuse University
Temple University
Tufts College
Union College
University of Chicago
University of Chicago Medical School
University of Cincinnati
University of Illinois
University of Iowa
University of Missouri
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Rochester Medical School
University of Vermont
University of Virginia
University of Wisconsin
Vanderbilt University
Vanderbilt University Medical School
Vassar College
Wabash College
Wellesley College
Wesleyan University
Western Reserve University
Wheaton College
Wilson College
Wistar Institute of Anatomy and Biology
Yale University

5. EVENING LECTURES, 1935

Friday, June 28	Dr. W. J. V. Osterhout	“How Do Electrolytes Penetrate the Cell?”
Friday, July 5	Mr. Columbus Iselin	“The Current System of the Western North Atlantic.”
Friday, July 12	Dr. E. F. Adolph	“Control of the Rate of Water Excretion in the Frog Kidney.”
Friday, July 19	Dr. T. Hume Bissonnette	“Sexual Photoperiodicity.”
Tuesday, July 23	Dr. Albert Navez	“Effects of Growth-promoting Substances in Plants.”
Friday, July 26	Dr. Martin Kilpatrick	“Acid and Basic Catalysis.”
Friday, August 2	Lt. Commander E. H. Smith	“The Work of the International Ice Patrol as Carried Out by the United States Coast Guard.”
Friday, August 9	Dr. Paul Weiss	“The So-called Resonance Principle of Nervous Control Revised.”
Friday, August 16	(1) Dr. E. G. Conklin	“The History of Woods Hole and of the Marine Biological Laboratory.”
	(2) Dr. B. R. Coonfield	“Motion Pictures Illustrating the Present Activities of the Marine Biological Laboratory.”
Thursday, August 22	Dr. R. E. Cleland	“The Evening Primrose (Œnthera)—a Cytogenetic Non-conformist.”
Friday, August 30	Dr. Hugh M. Smith	“Zoölogical Observations and Experiences during Twelve Years in Siam.”

6. SHORTER SCIENTIFIC PAPERS, 1935

Tuesday, July 2	Dr. C. C. Speidel	“Effects of Alcohol on Nerves.”
	Dr. Roberts Rugh	“Ovulation in the Frog.”
Tuesday, July 9	Dr. Dugald E. S. Brown	“The Liberation of Energy during the Simple Twitch of Skeletal Muscle.”

Dr. W. Wilbrandt	"The Effect of Organic Ions on the Nerve Membrane Potential."
Dr. Kenneth C. Fisher and Dr. Laurence Irving	"Respiratory Poisons and the Frequency of the Embryonic Fish Heart."
Dr. W. E. Garrey	"Respiratory Metabolism during Cardiac Inhibition."
Dr. Laurence Irving	"The Respiratory Metabolism of the Seal."

Tuesday, July 16

Dr. Ethel Browne Harvey	"Some Surface Phenomena in Centrifuged Sea Urchin Eggs."
Dr. D. P. Costello	"The Effects of Centrifuging the Eggs of Nudibranchs."
Dr. L. Monné	"The Permeability of the Nucleus of Amœba to Dyestuffs."
Dr. Robert Chambers	"Cortical Changes in Cell Division."

Tuesday, July 30

Dr. L. G. Barth	"Quantitative Studies on the Rate of Regeneration of Tubularia."
Dr. B. H. Willier, Dr. R. F. Gallagher and Dr. F. C. Koch	"Sex-modification in the Chick Embryo Resulting from Injections of Male and Female Sex-hormones."
Dr. Paul S. Galtsoff	"Physiology of Ovulation and Ejaculation in the Oyster."

Tuesday, August 6

Dr. A. K. Parpart	"The Permeability of the Erythrocyte to Heavy Water."
Dr. Oscar W. Richards	"Killing Organisms with Chromium as from Incompletely Washed Bichromate-sulfuric Cleaned Glassware."
Dr. C. V. Smythe	"The Effect of Oxygen Consumption on Alcoholic Fermentation in Yeast Extracts."
Dr. J. K. W. Ferguson	"The Scope of the Chemical Method of Estimating Hb-CO_2."
Dr. Shiro Tashiro	"Dacryorrhetin: the Demonstration of Its Action by a Motion Picture."

Tuesday, August 13

Dr. P. S. Henshaw	"Biological Factors Influencing the Radiosensitivity of Cells."
Dr. Henry J. Fry	"The Significance of Mid-bodies."
Dr. J. G. Carlson	"The Intergeneric Homology of a Euchromosome in Several Closely Related Acridinæ."

Dr. C. E. McClung	"Phylogenetic Significance of Some Structural Conditions in the Orthoptera."

Tuesday, August 20

Dr. William Trager	"On the Nutritional Requirements of Mosquito Larvæ."
Dr. J. E. Davis	"The Experimental Production of Tumors in Rats through Cellular Malnutrition."
Dr. Leonard B. Clark	"Dark Adaptation in the Insect Dineutes assimilis."
Dr. L. V. Heilbrunn	"Protein Lipid Binding in Protoplasm."
Dr. Paul S. Galtsoff	"The Effect of Crude Oil Pollution on Marine Life."

Tuesday, August 27

Dr. H. Burr Steinbach	"Diffusion Potentials in Biological Systems."
Dr. W. J. V. Osterhout	"The Rôle of Ions in Valonia and Nitella."
Dr. M. H. Jacobs and Dr. Dorothy R. Stewart	"The Manner of Entrance of Ammonium Salts into Cells."
Dr. E. Torsten Teorell	"Some Aspects of Electrolyte Diffusion."

Thursday, August 29

Dr. Robert Chambers	"Studies on the Physical Properties of the Plasma Membrane."
Dr. Hope Hibbard and Dr. Robert Chambers	"Micromanipulation of Egg and Nurse Cells in Bombyx mori."
Dr. Ethel Browne Harvey	"Parthenogenetic Merogony or Cleavage without Nuclei in Arbacia punctulata."
Mr. L. Churney	"The Quantitative Determination of Mitotic Elongation."
Dr. Donald P. Costello	"The Hyaline Zone of the Centrifuged Egg of Nereis."
Mr. Floyd Moser	"Changes at the Surface of Arbacia punctulata Eggs during Membrane Elevation."
Dr. Heinz Holter	"Localization of Peptidase Activity in the Eggs of Echinarachnius and Arbacia."
Dr. E. Alfred Wolf and Mr. T. F. McBride	"Early Stages of Ossification."
Mr. T. F. McBride and Dr. E. Alfred Wolf	"Early Stages of Calcification in Teeth."

Dr. E. R. Clark, Mrs. Eleanor Linton Clark and Dr. R. O. Rex	"Observations on the Behavior of Polymorphonuclear Leucocytes as Seen in the Living Animal."
Dr. Elbert C. Cole	"Methylene Blue Staining Sequences in the Walls of the Digestive Tube of the Frog." "The Effects of Ethyl Carbamate and of Potassium Cyanide upon the Staining Capacity of Ganglion Cells and of Cells of Smooth Muscle Type."
Dr. R. A. Budington	"The Tolerance of Acetyl-salicylic Acid by Sperm and Eggs of Arbacia."
Dr. Louise Palmer	"The Shedding Reaction in Arbacia."
Dr. H. H. Plough	"The Individual Specificity of Blood in Inhibiting Self-fertilization in Three Species of the Ascidian, Styela."
Dr. B. H. Grave and Mr. Jay Smith	"Sex Inversion in Teredo and Its Relation to Sex Ratios."
Dr. Walter N. Hess	"Reactions to Light and the Photoreceptors in Dolichoglossus kowalevskyi."
Mr. James A. Donnellon	"An Experimental Study of Clot Formation in the Perivisceral Fluid of Arbacia."
Dr. Manton Copeland	"Keeping Nereis for Physiological Study."
Dr. Francis Leövey	"Changes in Osmotic Pressure of Teleost Muscle as a Result of Changes in External Salt Concentration (with special reference to the Tautog)."
Dr. E. F. Adolph	"Osmotic Exchanges of Phascolosoma."
Dr. W. A. Dreyer	"Water Content of Insects in Relation to Temperature and Humidity."
Mr. Millard W. Bosworth and Dr. William R. Amberson	"An Adaptation of the Manometric Van Slyke Apparatus to the Study of the Respiration of Marine Animals."

Mr. John Stanbury and
Dr. William R. Amberson ...... "The Rate of Regeneration of the Plasma Proteins after Complete Exsanguination."

Dr. Charlotte Haywood and
Dr. Rudolf Höber .... "The Permeability of the Frog Liver to Certain Lipoid Insoluble Substances."

Dr. M. E. Krahl and
Dr. G. H. A. Clowes .... "Effect of Nitrophenols and Related Compounds on Metabolism of Living Cells."

Dr. G. H. A. Clowes,
Dr. M. E. Krahl and
Miss Anna K. Keltch .... "Stimulation and Depression of Respiration in Relation to Cell Division."

Dr. A. E. Navez and
Dr. Ethel Browne Harvey ...... "Indophenol Oxidase Activity in Intact and Fragmented Arbacia Eggs."

Miss Rita Guttman .... "Differential Oxygen Uptake of Regions of Limulus Optic Nerve as Related to Distance from the Sense Organ."

Dr. Walter S. Root .... "Lactic Acid in Dogfish Nerve."

Dr. Emil Bozler .... "Mechanical Properties of Smooth Muscles."

Dr. A. K. Parpart .... "A Method for Measuring the Intercellular Spaces in Tissues."

Dr. F. J. M. Sichel and
Dr. C. Ladd Prosser .... "Spatial Relations in the Excitation of the Isolated Muscle Fibre."

Dr. M. H. Jacobs and
Dr. A. K. Parpart .... "The Permeability of the Erythrocyte to Ammonia."

Mr. F. R. Hunter and
Dr. E. Newton Harvey .... "The Effect of Lack of Oxygen on the Permeability of the Egg of Arbacia to Ethylene Glycol." (Read by title.)

DEMONSTRATIONS

Thursday, August 29

Dr. Ethel Browne Harvey ..... "Demonstration of Cleavage without Nuclei."

Mr. William E. Williams and
Dr. Elbert C. Cole .... "Permanent Preparations of Vertebrate and Invertebrate Tissues Stained with Phosphate-hæmatoxylin."

Friday, August 30

Dr. Emil Bozler	"Significance of Calcium for Contractility of Vertebrate Smooth Muscle."
Dr. E. R. Clark and Mrs. Eleanor Linton Clark	"The Microscopic Study of Living Tissues in the Mammal."
Dr. Leonard B. Clark	"Apparatus to Measure the Dark Adaptation and Intensity Discrimination of the Fiddler Crab as Related to the Visual Field."
Dr. Manton Copeland	"A Tetrad for the Lecture Desk."
Dr. Heinz Holter	"Micro-titration Apparatus."
Dr. Louise Palmer	"Shedding Reaction in Arbacia."
Dr. A. K. Parpart	"Apparatus for Measuring Intercellular Fluid." "A Method for Showing Swelling and Shrinking of Erythrocytes."
Dr. Alexander Sandow	‘Diffraction Spectra of Striated Muscle."
Dr. H. Shapiro	"Capillary Microrespirometer, after Gerard and Hartline."
Dr. Eleanor H. Slifer	"One Hundred and Thirty New Organs in the Grasshopper."
Dr. Torsten Teorell	‘An Arrangement for Studying the Conditions within Diffusion Layers."

7. MEMBERS OF THE CORPORATION

1. Life Members

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Maryland.
Billings, Mr. R. C., 66 Franklin St., Boston, Massachusetts.
Conklin, Prof. Edwin G., Princeton University, Princeton, New Jersey.
Crane, Mr. C. R., New York City.
Evans, Mrs. Glendower, 12 Otis Place, Boston, Massachusetts.
Fay, Miss S. B., 88 Mt. Vernon St., Boston, Massachusetts.
Foot, Miss Katherine, Care of Morgan Harjes Cie, Paris, France.
Gardiner, Mrs. E. G., Woods Hole, Massachusetts.
Jackson, Miss M. C., 88 Marlboro St., Boston, Massachusetts.
Jackson, Mr. Chas. C., 24 Congress St., Boston, Massachusetts.
Kidder, Mr. Nathaniel T., Milton, Massachusetts.
King, Mr. Chas. A. ,
Lee, Mrs. Frederic S., 279 Madison Ave., New York City.

Lowell, Mr. A. Lawrence, 17 Quincy St., Cambridge, Massachusetts.
McMurrich, Prof. J. P., University of Toronto, Toronto, Canada.
Means, Dr. James Howard, 15 Chestnut St., Boston, Massachusetts.
Merriman, Mrs. Daniel, 73 Bay State Road, Boston, Massachusetts.
Minns, Miss Susan, 14 Louisburg Square, Boston, Massachusetts.
Morgan, Mr. J. Pierpont, Jr., Wall and Broad Sts., New York City.
Morgan, Prof. T. H., Director of Biological Laboratory, California Institute of Technology, Pasadena, California.
Morgan, Mrs. T. H., Pasadena, California.
Noyes, Miss Eva J.
Phillips, Mrs. John C., Windy Knob, Wenham, Massachusetts.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
Sears, Dr. Henry F., 86 Beacon St., Boston, Massachusetts.
Shedd, Mr. E. A.
Thorndike, Dr. Edward L., Teachers College, Columbia University, New York City.
Trelease, Prof. William, University of Illinois, Urbana, Illinois.
Ware, Miss Mary L., 41 Brimmer St., Boston, Massachusetts.
Wilson, Dr. E. B., Columbia University, New York City.

2. Regular Members, 1935

Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts.
Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
Adolph, Dr. Edward F., University of Rochester Medical School, Rochester, New York.
Allee, Dr. W. C., The University of Chicago, Chicago, Illinois.
Allyn, Dr. Harriet M., Mount Holyoke College, South Hadley, Massachusetts.
Amberson, Dr. William R., University of Tennessee, Memphis, Tennessee.
Anderson, Dr. E. G., California Institute of Technology, Pasadena, California.
Armstrong, Dr. Philip B., Cornell University Medical College, 1300 York Avenue, New York City.
Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts.
Baitsell, Dr. George A., Yale University, New Haven, Connecticut.
Baldwin, Dr. F. M., University of Southern California, Los Angeles, California.

Ball, Dr. Eric G., Johns Hopkins Medical School, Baltimore, Maryland.

Bard, Prof. Philip, Johns Hopkins Medical School, Baltimore, Maryland.

Barron, Dr. E. S. Guzman, Department of Medicine, The University of Chicago, Chicago, Illinois.

Barth, Dr. L. G., Department of Zoölogy, Columbia University, New York City.

Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York.

Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, Louisiana.

Bennitt, Dr. Rudolf, University of Missouri, Columbia, Missouri.

Bigelow, Dr. H. B., Museum of Comparative Zoölogy, Cambridge, Massachusetts.

Bigelow, Prof. R. P., Massachusetts Institute of Technology, Cambridge, Massachusetts.

Binford, Prof. Raymond, Guilford College, Guilford, North Carolina.

Bissonnette, Dr. T. Hume, Trinity College, Hartford, Connecticut.

Blanchard, Prof. Kenneth C., New York University, Washington Square College, New York City.

Bodine, Dr. J. H., University of Iowa, Iowa City, Iowa.

Boring, Dr. Alice M., Yenching University, Peking, China.

Bowling, Miss Rachel, Department of Zoölogy, Columbia University, New York.

Box, Miss Cora M., University of Cincinnati, Cincinnati, Ohio.

Bradley, Prof. Harold C., University of Wisconsin, Madison, Wisconsin.

Bridges, Dr. Calvin B., California Institute of Technology, Pasadena, California.

Bronfenbrenner, Dr. Jacques J., Department of Bacteriology, Washington University Medical School, St. Louis, Missouri.

Bronk, Dr. D. W., University of Pennsylvania, Philadelphia, Pennsylvania.

Brooks, Dr. S. C., University of California, Berkeley, California.

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THE MEMBRANES AND GERMINAL VESICLE OF THE EGG OF SABELLARIA VULGARIS

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I

The newly-shed egg of *Sabellaria* is very irregular in shape and is enclosed by a rather thick vitelline membrane which is discontinuous with the egg protoplasm. Immediately after shedding this membrane elevates from the egg surface, the egg becomes spherical and, at the same time, a second or hyalin plasma membrane appears on the egg surface. Since the stimulus for membrane elevation seems to be the contact with sea water, the alterations undergone by these membranes during and after elevation have been examined in the light of those described for the membranes of other eggs after sperm entrance. The alterations restrict the time during which sperm penetration can take place.

A preliminary study has also been made of the free germinal vesicle of this egg. The vesicles can be secured within about eight minutes after shedding, by placing the eggs in distilled water. The behavior of the vesicles in hypo- and hypertonic sea water, in various salt solutions, and when exposed to certain vital dyes and indicators has been examined. The free vesicle does not go through any prematuration change; it will persist for hours in solutions which destroy the rest of the egg and sperm do not react to its presence.

II

Shedding is by way of the nephridiopores and is provoked by internal muscular activity, as well as by rhythmic contraction and elongation of the animal. After shedding, the contractions cease and the animal becomes quiescent.

As the eggs emerge from the nephridiopores they are of various shapes, caused by pressure in the coelomic cavities and during shedding; the endoplasmic granules are unevenly distributed; the germinal vesicle is large and somewhat irregular in outline; and in general the eggs appear quite light in color. Frequently the deformation is so pronounced that the wall of the germinal vesicle is in contact with that of the egg. No trace of the original region of attachment of the egg to the ovary can be seen, nor is there any indication in the newly shed egg of the future animal pole. As the eggs emerge singly and the vitelline membrane is at first somewhat sticky, they adhere in long strings. Elevation and hardening of the membrane, rounding up of the eggs and movement of the female soon break up the strings and the eggs become loosely scattered on the bottom of the container.

After shedding several other changes can be seen to occur within the egg. As the eggs assume a spherical shape, the endoplasmic granules become more evenly distributed so that the eggs are no longer light in appearance, but are packed with granules which obscure the outline of the germinal vesicle. Because of the irregular shape of the eggs when first liberated, it has not been possible to determine if any change in volume occurs after shedding. The germinal vesicle becomes rounded and then irregular again and appears to move very slowly towards one side of the egg, the future animal pole. The irregular vesicle, or its derivative, continues this migration and eventually forms a clear, finely granular area, free from the larger granules, at the animal pole.

III

The Vitelline and Hyalin Plasma Membranes

Within the first few minutes after shedding the vitelline membrane elevates from the egg surface. At first the elevation is quite uniform and the membrane appears smooth and without wrinkles, but as elevation proceeds it becomes irregular and abundantly wrinkled. This may be the result of differences in tension of the protoplasmic processes. The deeper indentations may extend to the egg surface (Fig. 1). This state persists generally for a long time and it is only in very old eggs or after fertilization when these processes are withdrawn, that the wrinkles disappear. The outline of the membrane then becomes slightly oval rather than spherical and the space between membrane and egg is enlarged.

Coincident with the elevation of the vitelline membrane, a thin layer of transparent hyalin material of uniform thickness, the hyalin plasma membrane, appears on the surface of the egg. From the egg surface, numerous fine protoplasmic strands or threads can be seen to extend towards the vitelline membrane and they have been traced to the membrane itself. These persist until fertilization, and may be concerned in transmitting the stimulus of sperm contact across the subvitelline space. They disappear following centrifuging. It remains to be seen if lightly centrifuged eggs from which the strands have disappeared can be fertilized. The few preliminary experiments of this kind have not been successful. Hobson (1932) describes protoplasmic strands in the egg of *Teredo norvegica* which are pulled out from the egg surface during the separation of the vitelline membrane. These may be slender threads or broad flame-shaped processes. This is followed by the slow retraction of the strands which seem as though composed of a viscous fluid. If a strand breaks during retraction each half contracts, one to the egg surface where it disappears and the other to the membrane surface.

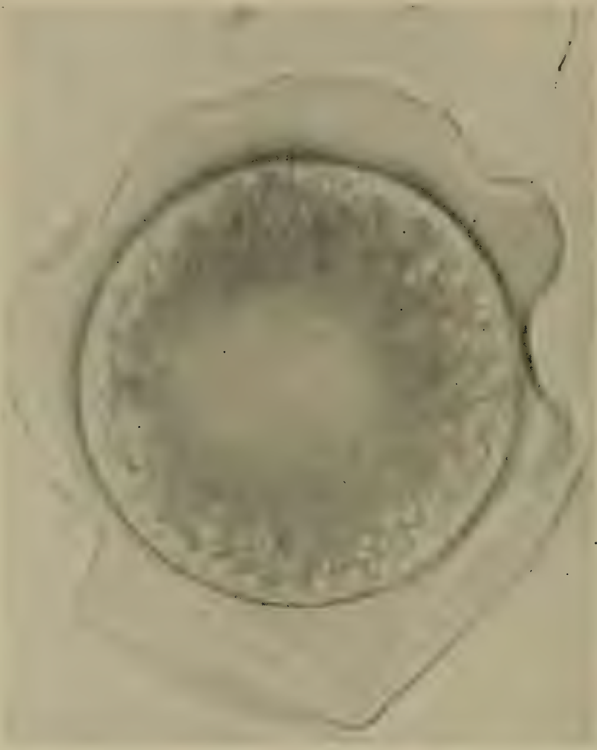

FIG. 1. Photomicrograph of a newly-shed egg. The elevated wrinkled vitelline membrane, the fine protoplasmic strands extending from the egg surface towards the membrane, and the extension of the germinal vesicle material toward one side of the egg are visible.

In *Sabellaria* the strands are likewise of a viscous nature, but they are very fine and numerous and appear to be pulled out by the elevation of the vitelline membrane (Fig. 1). The material of the hyalin plasma layer seems to form between and around their bases. The strands are more permanent than those described by Hobson. They persist for hours in old eggs and withdrawal occurs after sperm entrance, centrifuging, treatment with hypotonic sea water or distilled water, etc. During retraction following fertilization, they do not break but become shorter, thicker, beaded or knobbed and finally disappear within the egg surface. The strands are evidently under some tension and the material of which they are composed is elastic. After their withdrawal the hyalin plasma membrane becomes a uniform layer. During the elevation of the fertilization cone, the strands involved become lost in the substance of the cone. No separate fertilization membrane appears after sperm entrance.

The vitelline membrane of the sabellarian egg appears to be a preformed structure (*cf.*, Fauré-Fremiet, 1924). In stained sections of the female, it is visible as a very thin but distinct membrane enclosing the unshed eggs as they lie in the coelomic cavity. It may also be seen to encircle the developing oöcytes except where they are attached to the ovary. At this time the membrane is thin but gradually it increases in thickness as development proceeds. It seems to be discontinuous with the egg cytoplasm. In freshly-shed eggs, it is slightly thicker as some swelling occurs on contact with the sea water, but during elevation it becomes thinner as it is stretched. At first the membrane is soft, somewhat gelatinous, elastic and slightly sticky. Tension cannot be very great or the eggs would approach more towards a spherical shape while in the abdominal cavity of the female.

The plasticity of the membrane changes after exposure to sea water. The change in the shape of the egg is a surface phenomenon involving a permeability change and is not influenced directly by the vitelline membrane, though if elevation is retarded, the rounding up of the eggs takes place more slowly. Elevation occurs simultaneously and in a somewhat uniform manner over the entire egg surface, not starting at one point (sperm contact-point) as is the case with fertilization membranes. During fertilization the force of sperm entrance may pull the membrane towards the egg, but after the sperm-head is through, it flips outward again to its original position.

After contact with sea water, the vitelline membrane hardens, as shown by placing the eggs at different times after shedding into distilled water and noting the rate and the numbers of the eggs which swell and cytolyze accompanied by further elevation and rupture of the membrane (see Moore, 1930; Hobson, 1927; and Costello, 1935 for fertilization membranes). For example, eggs shed from 2:24–2:25 P.M. were transferred at one-minute intervals to plain distilled water. At this time the eggs were spherical and the membrane had elevated.

In eggs transferred within the first five minutes cytolysis occurred immediately and the membrane elevated until it broke. It quickly vanished and the cytoplasm, except for the vitelline granules, disappeared leaving naked swollen germinal vesicles. In eggs transferred during the next seven minutes a progressively increasing number of the membranes failed to rupture immediately, and they persisted for longer intervals. The membranes of eggs transferred at 2:38 persisted without rupture or other change for hours.

This, together with other experimental data, indicates a pronounced hardening of the vitelline membrane with loss of solubility and elasticity during the first twelve minutes after shedding. The rate of cytolysis was much the same in the several cultures, showing that there was little change in the permeability of the egg and membrane to water during the period. The amount of hardening during the first hour at least is not sufficient to inhibit sperm entrance, but there may be some retarding effect since the highest percentage of fertilization has been secured when eggs and sperm were shed together in the same container. At the time of shedding the membrane is quite soft and, as seen above, it is readily soluble in distilled water. This physical change in the membrane may account in part for the very low percentage of fertilization secured in eggs which have stood for longer than one hour.

The membranes which persist for some time after escape of the contents are irregular in outline and markedly thinner than those of normal eggs. This shows that the preformed membrane is capable of considerable extension in newly-shed eggs, as has been demonstrated by Just (1928) for the unfertilized egg of *Arbacia* and by Chase (1935) for the egg of *Dendraster*. Moreover, the membranes become rigid, fail to collapse and the ruptured edges do not curl or stick to the membranes of adjacent eggs with which they come in contact.

The physical changes occurring in the hyalin plasma membrane, following its first appearance, resemble in several respects those of the vitelline membrane. At first it is thin, gelatinous, and quite soft, offering no resistance to the swelling and bursting of the egg in distilled water. It is weaker than at a later stage. After the elevation of the vitelline membrane, the plasma membrane increases in thickness. Since it is not visible before elevation and its appearance is not dependent on fertilization, it may be the result of the reaction of the exposed cortical cytoplasm of the egg. Within one-half hour after shedding, there is a noticeable stiffening and loss of elasticity on the part of the membrane. It slowly becomes fairly tough, rigid and less soluble, but not to the same extent nor as quickly as does the vitelline membrane. Because of this alteration a longer time is required for the eggs to burst or they may not burst at all but form exovations. This progressive hardening of the hyalin plasma membrane continues slowly. When tested with distilled water the older eggs cytolyze quickly, swell slightly, but only a few burst even after several hours of exposure.

Harvey (1934) describes the hyalin plasma membrane of fertilized sea-urchin eggs as a thin colorless layer which appears on the surface of the egg after fertilization. Ten to fifteen minutes after fertilization it thickens. It is an extracellular membrane rather than an integral part of the cell protoplasm. The observations of Harvey would seem to support the conclusion that the appearance of the plasma membrane coincides with the elevation of the vitelline membrane irrespective of what initiates the elevation of the latter. Moore (1928) considers it to be a secretion membrane. He finds that the material, of which this membrane is made, is secreted by the fertilized egg, blastomeres, blastulæ and early gastrulæ, but not by advanced gastrulæ and plutei. The addition of Ca to sea water renders this membrane relatively insoluble and inelastic.

Unlike the results of Moore (1935) in the case of the eggs of certain echinoids, isosmotic glycerine and urea (pH close to that of sea water) do not seem to inhibit membrane elevation in freshly shed eggs. Instead there is an acceleration of elevation in these non-electrolyte solutions and the membrane rapidly disappears. When returned to fresh sea water, the eggs which did not cytolyze and disintegrate underwent the prematuration changes of normal unfertilized eggs. Eggs freed of their vitelline membranes by the above method exhibited marked constancy of form and size in fresh sea water, as revealed by camera lucida outline drawings at frequent intervals. No processes were present.

These eggs are capable of being fertilized. No elevation of any secondary membrane was seen to follow sperm entrance. The cleavages were typical as far as they were followed and the blastomeres formed the compact cellular mass characteristic of normal development. From the above observations it is difficult to imagine any other function for the vitelline membrane of this animal egg than that of protection to the developing embryo. Its early elevation forms no obstruction to sperm entrance, possibly aided by the presence of protoplasmic processes which traverse the subvitelline space and transmit the stimulus of sperm contact. The absence of these processes in membraneless eggs offers no obstruction to fertilization.

In other experiments, eggs shed into isosmotic glycerine (pH about 7.0) were transferred at one-minute intervals thereafter to fresh sea water. The eggs returned to normal size and membranes elevated in cultures transferred up to five minutes after shedding. The numbers elevating membranes steadily decreased during this interval. Eggs transferred after five minutes exposure formed no membranes during the course of an hour. The remaining eggs in the glycerine were transferred after thirteen minutes exposure to distilled water. Entire disintegration occurred, except in the case of the germinal vesicles. A more acid glycerine solution, with a pH of about 6.0, gave different results from those just described. The membranes elevated as before, though a trifle more slowly, and instead of disappearing, persisted for about an hour. Thus a more acid glycerine preserves the vitelline membrane for some time but does not prevent its elevation. In general eggs shed into glycerine show a marked tendency to clump or stick together.

Chase (1935) has studied the solubility of egg membranes in an isosmotic solution of urea. After such treatment the unfertilized eggs of *Dendraster excentricus* and *Strongylocentrotus purpuratus* failed to form membranes and the fertilization membrane in the early stages of its formation also was soluble. Both the vitelline and fertilization membranes of *Patiria miniata* and *Urechis caupo* were insoluble but those of the latter could be dissolved off after fifteen to twenty-four hours exposure. In the present study the membranes elevated immediately before the glycerine had time to dissolve them. Afterwards they were dissolved but the time required increased with the age of the eggs.

The effect of ions upon the elevation of the vitelline membrane in glycerine was tested by adding varying amounts of M/60 $CaCl_2$, M/60 $MgCl_2$, M/40 NaCl or M/40 KCl to the glycerine. The eggs from different females were used for the study, and the eggs were shed directly into the solutions. The addition of $CaCl_2$ to the glycerine preserves the vitelline membrane and also retards its elevation. Potassium chloride accelerates membrane elevation while $MgCl_2$ retards elevation in the higher dilutions. The latter also retards rounding up of newly-shed eggs and the eggs clump together in large masses. Sodium chloride seems to have an accelerating effect upon membrane elevation but rounding up of the eggs is retarded. The few eggs which cytolyzed and burst, liberated free germinal vesicles which remained intact for hours in the solution.

The relative effect of these chlorides parallels that found by Lucké and McCutcheon (1932) in their investigation of the effect of ions upon the water permeability of sea-urchin eggs. When dissolved in a hypotonic solution of dextrose, the chlorides of Na, K, Li, or NH_3 increased the permeability to water more than in pure dextrose. When the chlorides of Ca or Mg were used, the permeability decreased to a point near that of sea water. According to Fauré-Fremiet (1924) the vitelline membrane of the egg of *Sabellaria alveolata* is permeable to electrolytes, crystalloids and dyes. Its elevation is not due to the osmotic pressure of the peri-ovular fluid but to the fact that the electric charge on the inner face of the membrane is the same as that on the surface of the egg. After the elevation of the vitelline membrane, the spherical egg is without any trace of another membrane.

IV

Germinal Vesicle

The vesicle of the newly-shed egg is a large, clear, irregular-shaped structure containing a single eccentrically located nucleolus and a few large granules in a fluid, finely granular nucleoplasm. No precise structure can be seen in it even when vitally stained. As the egg becomes spherical, the vesicle also rounds up but the distinctness of its outline is lost by the more uniform distribution of endoplasmic granules and globules. Shortly after this, it loses its spherical outline and the vesicle or its derivatives move toward one side of the spherical egg where a finely granular area appears at the animal pole. During this migration the outline of the vesicle undergoes a variety of changes in shape as shown by camera lucida outline drawings of individual eggs.

Any substance which produces cytolysis and sufficient swelling so that the egg membranes burst will give free naked germinal vesicles during a certain time after shedding, e.g., distilled water, hypotonic sea water and glycerine. In these solutions, swelling of the germinal vesicle occurs during cytolysis and swelling of the egg but not before. The osmotic equilibrium established at this time does not change even after long exposure to plain distilled water. There is little increase in size or other change after the swollen vesicles are freed from the remainder of the egg material and they remain intact for hours in hypertonic and hypotonic solutions, sea water, glycerine or distilled water. When transferred from distilled water to sea water or to a hypertonic solution, they shrink.

Several observations seem to point to the presence of an elastic solid surface or membrane in the free germinal vesicle which is in a state of tension. During and after swelling in distilled water the vesicle is spherical. The elastic solid layer is stretched but its elasticity is limited since occasionally it may rupture. The exovates are not soluble. In this case the vesicle proper decreases slightly as material is extruded, but the surface does not wrinkle. No thick structure or membrane surrounds the exovates as is the case with the vesicle itself.

In hypertonic sea water the free vesicle shrinks uniformly and with a smooth surface to about the normal diameter. With continued exposure it becomes crenated or even indented and with time this condition becomes more marked. Thus when water is removed by a hypertonic environment, the surface layer contracts, after which reduction of volume results merely in puckering or wrinkling. This stretching is not always reversible and the amount of contraction is variable in different vesicles from the same female. The layer contracts more in hypertonic salt solution than in sea water. Many swollen vesicles show little reduction in size when transferred to sea water, while others return to about normal size and do not pucker.

During swelling the vesicles practically double the original diameter but do not generally rupture no matter how long they are subsequently left in plain distilled water. They have been found twenty-four hours later with practically no change except for a clumping of the larger granules and globules at one side in the region of the nucleolus. Thus the surface layer as well as the contents of the vesicle, i.e., exovates, are insoluble, and this layer is quite tough, as swollen vesicles do not generally burst. Another observation is that vesicles, which have shrunk on transference to sea water and after an interval are placed again in distilled water, do not swell again or swell very little without rupture. This would seem to indicate a hardening and loss of elasticity of the surface layer after some exposure to sea water.

Fauré-Fremiet describes the germinal vesicle of *Sabellaria alveolata* as being about 55 μ. The contents are quite aqueous and a single acidophilic nucleolus is present. The germinal vesicle swells in strong concentrations of NaCl and $MgCl_2$, and, if the egg bursts, the vesicle is freed and will persist for many hours.

The free vesicle shows no specific internal structure with vital dyes. A large persistent nucleolus is present as well as a small number of large, irregular-shaped granules of various sizes. The ground substance seems to be a finely granular material. No evidence of any prematuration changes have been seen in the free vesicle even when transferred to sea water immediately after escaping from the cytolyzed egg. Vesicles which have stood in the solutions for some time, for example distilled water, show less reduction in size when returned to fresh sea water than those which are transferred soon after rupturing. It has been noticed that swelling of the vesicle accompanies egg swelling and cytolysis. Eggs which are slow to react to distilled water or glycerine do not show any preliminary enlargement or change in shape of the vesicle.

Free germinal vesicles can be secured from eggs up to about nine minutes after shedding. The greatest number appear from eggs shed directly into distilled water or placed in it during one to four minutes after shedding. Prematuration changes start around seven to eight minutes after shedding, so the vesicles secured after this time represent those which were somewhat delayed. In isosmotic glycerine, prematuration does not take place and the vesicles persist. If rupture does not occur here, free vesicles can be secured by transfer of these eggs to distilled water.

The wall of the germinal vesicle is permeable not only to water and perhaps glycerine, but also to certain dyes. The dyes employed include methyl violet, orange G, acid fuchsin, chrysoidin, methyl red, neutral red, methyl green, methylene blue, nile blue sulphate, and indicators as brom thymol blue, cresol red, thymol blue, and phenolphthalein. The germinal vesicles were transferred immediately after their liberation to fresh sea water and a dilute solution of the dye added.

Methyl violet in distilled water penetrated the isolated vesicle at once and gave a faint pinkish violet, with a pH above 5. Some of these eventually formed exovates which were stained, showing that the dye penetrated the vesicle wall. The cytolyzed egg cytoplasm stained a blue violet color, at a pH between 2.0 and 5.0. When transferred to sea water the vesicles became a light violet. The cytoplasm of normal unfertilized eggs of the same age were colored a deep blue violet which, together with evidence from other dyes and indicators, shows a pH above 5.0 and probably around the neutral point for the living egg. After transfer to distilled water, these eggs ruptured but the free germinal vesicle was without any trace of color. The depth of color of the free vesicle varies slightly with the concentration of the dye. Those in fresh sea water color a light violet, indicating a pH of 6.0 or above. The pH of sea water was around 8.2 and that of the distilled water 5.8. This dye stains the vitelline membrane a light pinkish violet.

In distilled water brom thymol blue colored the vesicle a light yellow, pH not above 6.0, while in sea water the color was a yellowish blue, showing a pH value between 6.0 and 7.0. These results would seem to indicate a variation of the internal pH of the free vesicle in harmony with that of the external medium. Orange G, acid fuchsin, and chrysoidin gave little, if any, differential staining. Methyl red stained the vesicle in sea water a yellowish red, indicating an internal pH around 6.0. Methylene blue is rapidly reduced by the vesicle.

Neutral red seemed to penetrate the vesicle quite slowly. It showed a densely granular structure with irregular clumping of the larger granules and globules. The red color indicated a pH below 7.0. This dye also penetrated the vitelline membrane slowly. Methyl green and methylene blue stained the granules. Nile blue sulphate entered readily and immediately gave a dark blue color even when noncytolyzed eggs showed no coloration. The dye seemed to enter with equal facility and to almost the same degree whether distilled water or fresh sea water constituted the external medium. Intact but cytolyzed eggs did not stain during the hour of observation.

In neutral red, methyl violet, methyl red and Nile blue sulphate, both the wall and contents of the germinal vesicle are stained. This is shown by the many cases in which colored exovations formed after the vesicle had stood for some time. Neutral red penetrated the vesicle membrane more slowly than any of the dyes employed. After an hour they were only slightly colored.

The above experiments represent only an initial study of the action of dyes on the free germinal vesicle. They would seem to indicate an internal pH value of the free germinal vesicle in fresh sea water between 6.0 and 7.0. In distilled water and sea water which are acid, the pH of the vesicle tends toward the acid side. In neutral distilled water or sea water the pH is near the neutral point. It is possible that these results have no real significance because the vesicle is not in its normal environment and furthermore it may be fatally injured by the method used in freeing the vesicle. If the phenomenon of cytolysis can be said to occur in the germinal vesicle freed by distilled water, then it must be expected (Needham, 1931) that it would appear more acid than is normally the case.

Needham (1931), in the discussion of the action of vital dyes on whole eggs, considers that the intracellular reaction is near the neutral point and that cytolysis is an acid-producing process. Injection of neutral red into the egg of *Sabellaria alveolata* gave a pH of 6.6. For this same egg Fauré-Fremiet (1924) found a pH less than 7.0 at zero to five minutes after shedding. This gradually increased to 12.0 or higher at forty to fifty minutes after shedding. If fertilized, there was an increase to 10.4 or 11 and then a fall to around 7.0 during the first stages of segmentation. Needham criticises these results as given by dyes which are not good pH indicators even under the best of conditions and which could not be trusted in a medium containing fatty substances for which they have a special affinity.

Needham also gives the results of Reiss, who found that in the unripe eggs of *Sabellaria:* (1) bromcresol purple gave a deep mauve for the germinal vesicle and yellow for the cytoplasm, and (2) bromthymol blue gave yellow for the cytoplasm, yellowish green for the germinal vesicle and bluish green for the nucleolus. The micro-compression method gave a pH of 5.0 for *Sabellaria* but this might be the result of injury (Needham). Like the results just described, he found that the pH of the nucleus was markedly affected by the pH of the external medium and varied in harmony with it. The pH of the intact vesicle, as found by Reiss (*cf.*, Needham, 1931), is very slightly higher than that found in the case of the isolated vesicle which was more toward the acid side.

In the present study it was noticed that the dye penetrated the germinal vesicle if it was free, but the vesicle of intact normal eggs did not generally stain during the experimental period, since after rupturing, the vesicles of stained eggs were colorless. The vesicle of cytolyzed and swollen eggs did stain. Monné (1935) has studied the permeability of the nuclear membrane of two *Amœba* species to vital stains. He used some forty dyes and sprayed them against the nuclear membrane with a micropipette. Immersion of the whole *Amœba* in the dyes failed. The living cytoplasm of the normal sabellarian egg, as well as that of the *Amœba,* acts as a barrier to dye penetration into the nucleus when sublethal concentrations of the dye are used. If the egg is killed, then certain dyes will enter and stain diffusely.

SUMMARY

The vitelline membrane of the egg of *Sabellaria vulgaris* is a preformed structure whose elevation is stimulated by exposure to sea water. During and after its elevation a second or hyalin plasma membrane forms. During the first hour after shedding these membranes undergo certain physical alterations which probably are responsible in part for the very low percentage of successful sperm penetration. These alterations are described as well as the effect of a non-electrolyte solution and various salts on membrane elevation.

The behavior of the free germinal vesicle of this egg in sea water, hypo- and hypertonic solutions is also described. The vesicle, freed of any of the other egg materials, can be secured within about eight minutes after shedding, by placing the eggs in distilled water. The penetration of certain vital dyes and indicators is discussed and also the information these reveal of the internal pH of the free vesicle.

LITERATURE CITED

Chase, H. Y., 1935. The origin and nature of the fertilization membrane in various marine ova. *Biol. Bull.*, **69**: 159.

Costello, D. P., 1935. Fertilization membranes of centrifuged Asterias eggs. I. The effects of centrifuging before fertilization. *Physiol. Zoöl.*, **8**: 65.

Fauré-Fremiet, E., 1924. L'œuf de Sabellaria alveolata L. *Arch. Anat. Micros.*, **20**: 211.

Harvey, E. B., 1934. Effects of centrifugal force on the ectoplasmic layer and nuclei of fertilized sea urchin eggs. *Biol. Bull.*, **66**: 228.

Harvey, E. N., 1910. The mechanism of membrane formation and other early changes in developing sea-urchin's eggs as bearing on the problem of artificial parthenogenesis. *Jour. Exper. Zoöl.,* **8**: 355.

Hobson, A. D., 1927. A study of the fertilization membrane of the echinoderms. *Proc. Roy. Soc. Edinb.,* **47**: 94.

Hobson, A. D., 1932. On the vitelline membrane of the egg of Psammechinus miliaris and of Teredo norvegica. *Jour. Exper. Biol.,* **9**: 93.

Just, E. E., 1929. Initiation of development in Arbacia. IV. Some cortical reactions as criteria for optimum fertilization capacity and their significance for the physiology of development. *Protoplasma,* **5**: 97.

Lucké, B., and M. McCutcheon, 1932. The living cell as an osmotic system and its permeability to water. *Physiol. Rev.,* **12**: 68.

Monné, L., 1935. Permeability of the nuclear membrane to vital stains. *Proc. Soc. Exper. Biol. and Med.,* **32**: 1197.

Moore, A. R., 1928. On the hyalin membrane and hyalin droplets of the fertilized egg of the sea urchin, Strongylocentrotus purpuratus. *Protoplasma,* **3**: 524.

Moore, A. R., 1930. Fertilization and development without the fertilization membrane in the egg of Dendraster excentricus. *Protoplasma,* **9**: 18.

Moore, A. R., 1933. The relative values of cations in protecting the membrane-forming capacity of the eggs of the echinoids, Clypeaster japonicus and Temnopleurus hardwickii. *Sci. Rep. Tohoku Imp. Univ., Sendai, Japan,* **8**: 249.

Needham, J., 1931. Chemical Embryology. Macmillan, N. Y.

Waterman, A. J., 1934. Observations on reproduction, prematuration, and fertilization in Sabellaria vulgaris. *Biol. Bull.,* **67**: 97.

ON THE ENERGETICS OF DIFFERENTIATION. III

COMPARISON OF THE TEMPERATURE COEFFICIENTS FOR CLEAVAGE AND LATER STAGES IN THE DEVELOPMENT OF THE EGGS OF SOME MARINE ANIMALS

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These experiments show principally that the temperature coefficients of the rate of attainment of the various cleavage stages and of the later developmental stages are identical for the eggs of some marine animals.

THEORETICAL PART

It has been concluded from earlier work (Tyler, 1933, 1935) that energy is required for the processes of embryonic differentiation, although the quantities involved could not be estimated. As part of a program to evaluate the energy requirements, an investigation of the temperature coefficients of the rate of development and of the rate of respiration was undertaken. This involved the determination of the temperature coefficients for cleavage for comparison with that of differentiation. In the previous work distinction was made between the maintenance, growth and differentiation processes of development. Cell division was left out of account. This was justified inasmuch as it did not enter as a factor in the results. In the cases of both the "half-embryos" and the giant embryos gastrulation occurs when the original egg has undergone the same number of cell divisions as it normally does. This was shown in the early work of Morgan (1901, 1903), Driesch (1900) and Bierens de Haan (1913). In considering the effect of temperature on development there is, of course, the possibility that cleavage and differentiation may be affected differently. That would mean that the embryos in the same stage of differentiation would contain different numbers of cells if reared at different temperatures, provided, of course, that the later divisions have the same temperature coefficient as the earlier ones.

The data that exist in the literature in regard to this point are of two sorts, namely, that in which estimates of the number of cells present in embryos of the same stage, but raised at different temperatures, are

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given, and that in which the temperature coefficients of the rate of cleavage and of attainment of the later stages are given. Data of the first kind are found in the work of Marcus (1906), Godlewski (1908), Boring (1909) and Koehler (1912), all of whom were interested in the effect of temperature on the nucleo-plasma ratio. The second kind of data is found in the work of Hertwig (1898), Peter (1905), Erdmann (1908), Krogh (1914), Ephrussi (1933) and Atlas (1935). With the exception of Boring, all of the first group find different numbers of cells in embryos of the same stage raised at different temperatures. In the second group only Atlas finds the same values for the temperature coefficients of the rate of cleavage and of attainment of the later stages.

Marcus, Godlewski and Koehler worked with the sea urchin, and although they all found larger numbers of cells in the embryos of the same stage raised at the higher temperatures, the relative values differed rather widely. Thus Marcus found the ratio of the number of cells in blastulæ at the beginning of mesenchyme formation to be 1 : 2.5 for the temperatures 9° and 22° C.; Godlewski obtained 1 : 1.81 for 12° and 21°; Koehler obtained 1 : 1.11 for 10° and 22°. Koehler claimed that the method employed by Marcus and Godlewski of counting the nuclei in the optical section was less accurate than his own method of counting the nuclei in a definite area of the surface of the embryo. Although he obtains values that are fairly close for the number of cells in blastulæ raised at different temperatures, in the later stages the differences are more marked. His figures for the early and late gastrula stages are:

Early gastrula	624 (10°),	820 (16°),	869 (22°)
Late gastrula	1040 (10°),	1163 (16°),	1595 (22°).

It may be noted that in the early gastrula stage the cell numbers for 16° and 22° are much closer than for 10° and 16°, whereas in the late gastrula the reverse is true. This it seems reflects on the accuracy of the results.

Miss Boring worked on the eggs of *Ascaris*. Her data are not very extensive, the counts being limited to groups of 3 to 5 cells of the surface ectoderm of the head region of embryos raised at 18° and 37° C. But she concludes that the number of cells is the same in embryos raised at the two different temperatures. The criticism of Erdmann (1908*b*) that the range of temperatures employed is outside of that for normal development cannot be significant, since Zawadowsky and Sidorov (1928) found that *Ascaris* eggs could develop at 7° C.

The temperature coefficient measurements of Peter gave higher values for cleavage than for the later stages in the case of the sea urchins, *Sphærechinus* and *Echinus*. Peter also calculated the coefficients for the frog from Hertwig's data and in this case the values for cleavage were lower than for the later stages. His Q_{10} figures are

Cleavage	2.29 (*Sphærechinus*)	2.30 (*Echinus*)	2.23 (*Rana*)
Later stages	2.03 (*Sphærechinus*)	2.08 (*Echinus*)	3.34 (*Rana*)

However, one can hardly attribute much significance to these figures since they are averages of values from widely different parts of the temperature scale, and as Peter himself shows, the Q_{10} at low temperatures is much higher than at high temperatures. Moreover, constant temperatures were not maintained, so that it is fruitless to try to compare the data for the later stages with cleavage even in a single experiment.

Krogh investigated the frog's egg at temperatures ranging from 2.7° to 22°. He chose five stages in the later development from closing of the medullary folds to the 7.8 mm. larva and found that the rates of attainment of these stages were similarly affected by temperature. He did not compare the values for the later stages with those for cleavage, but from his curves it can be seen that in the interval of 10° to 20° C. cleavage is speeded up 2.4 times, whereas the figure for the later stages is 3.6.

Ephrussi determined the time of first cleavage and of hatching of the blastula out of the fertilization membrane for the sea urchin at temperatures between 10° and 26° C. From his graphs (p. 85) it may be seen that cleavage is speeded up 3.1 times at 20° as compared with 10° and the rate of attainment of the hatching stage is speeded up 3.0 times. These values are evidently the same, but he draws the opposite conclusion from the data as a whole. Ephrussi considers hatching to represent a definite morphological stage (*cf*. p. 120). This is, however, not necessarily the case. In the trout embryo Gray (1928) finds that the morphological development at the time of hatching is different with different temperatures of incubation. My own experiments with *Ciona* show that embryos raised in concentrated cultures hatch out sooner than those in more dilute cultures. Thus for two cultures raised at 15° C., one containing about five hundred thousand embryos in 50 cc. of sea water, and the other about five thousand embryos in 50 cc., the following figures were obtained for the percentages of the embryos hatched at the indicated times after fertilization:

	Hours after fertilization						
	24.0	24.4	24.7	25.0	25.4	25.7	26
Concentrated culture	10	40	70	95	100	100	100
Dilute culture	0	0	0	10	40	80	95

The rate of cell division, however, was quite the same in both cultures. The earlier hatching of the crowded culture is very likely due to a greater concentration of the hatching enzyme which Berrill (1929) has shown to be produced by ascidian embryos.

Recently Atlas (1935) has found the temperature coefficients for the later stages of development of the frog's egg to be the same as for the cleavage stages over a considerable range of temperatures. Divergence began to appear above 20° C. but below that temperature the deviations are apparently within the limits of variability of the material.

It is evident from the lack of general agreement that further investigation of these points is desirable.

Experimental Part

Material and Methods

In these experiments the eggs of five marine animals were used; namely, the sand dollar, *Dendraster excentricus*, the sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus anamesus*, the ascidian, *Ciona intestinalis*, and the gephyrean worm, *Urechis caupo*. Only batches of eggs giving better than 95 per cent fertilization were followed, since there is the possibility that such factors as are responsible for the failure of a large percentage of the eggs to become activated may also affect the rate of development of the fertilized eggs. Also the disintegrating unfertilized eggs might at later stages affect the development of the others. In the great majority of the experiments, however, 100 per cent fertilization and 95 to 100 per cent normal development was obtained.

The temperature was controlled by means of a mercury-toluene thermoregulator operating through an a.-c. vacuum tube relay, and the variations were less than 0.01° C. For temperatures below that of the room, cold water was pumped from a refrigerated bath through coils of copper tubing in the constant temperature bath. This method was more satisfactory than direct immersion of the expansion coils of the refrigerator in the temperature bath, since the flow of cold water through the coils could be regulated so that it approximately balanced the warming-up of the bath.

The eggs were raised in flasks that were continuously shaken in the temperature baths. The rate of shaking was generally 50 round-trips per minute and the amplitude 3.5 cm. The flasks used were 125 cc. Erlenmeyers. They were filled with 50 to 60 cc. of sea water and contained in most runs a volume of eggs of about 5 cu. mm.

Two temperature baths were employed, so only two temperatures could be investigated at one time. The temperature was changed in only one bath at a time for new runs, leaving one to check any differences due to using eggs from different animals. Thus comparable data for all the temperatures employed could be obtained and the temperature-rate of development curves of Figs. 3 and 4 could be made. However, to compare the effects of temperature on the rate of cleavage and on the rate of attainment of the later stages it is sufficient to operate at two different temperatures at one time.

The eggs were either fertilized while in the temperature baths or introduced into the flasks in the baths at a definite time after fertilization. Since the flasks in the lower temperature baths would warm up temporarily upon the addition of an egg or sperm suspension of higher temperature, an appropriate amount of sea water cooled to the proper extent was, when necessary, added at the same time. This precaution is, of course, unnecessary except in cases of very low temperatures and when a large volume of sperm or egg suspension, in comparison with the volume of sea water in the flask, is added.

The time of attainment of a definite stage was determined by removing samples from the flasks at frequent intervals of time and either examining them immediately or preserving them for detailed examination. The time when 50 per cent of the developing eggs have reached a particular stage is given in the data, unless otherwise specified. For the cleavage stages the end-point chosen is that point at which the constriction of the cell first appears, for the later stages the end-points chosen are given in data presented.

The distribution curves for the attainment of a particular stage are not the same in relative magnitudes for the eggs of the various animals investigated. The eggs of *Ciona intestinalis* behaved most uniformly. As an example, the percentage of divided eggs found at various times after fertilization for a batch of eggs at 20° C. may be given.

	Minutes after fertilization					
	46¼	46½	46¾	47	47¼	47½
Per cent in 2 cells.............	0	10	20	50	85	100

The time difference between the first and last egg to divide is less than one minute for an average time of 47 minutes from fertilization. With the eggs of *Dendraster excentricus* the time difference was relatively greater as the following figures for a typical batch of eggs run at 20° C. show.

	Minutes after fertilization						
	53½	54	54½	55	55½	56	56¼
Per cent in 2 cells	0	5	20	50	75	95	100

The spread here is about 2¼ minutes for an average time of 55 minutes. *Urechis caupo* eggs gave a spread of about 2½ minutes for a first cleavage time of 76 minutes. *Strongylocentrotus purpuratus* eggs gave a 4-minute spread for a cleavage time of 77 minutes. For *Lytechinus* the figures at 15° are 7 and 137 minutes. At different temperatures the relative spread of the distribution curves will be the same since the time difference between the first and last egg to divide is increased or decreased by the same factor as the time for cleavage. It is obvious that sampling and counting errors have least effect where the relative spread of the distribution curves is smallest. Such errors, however, are slight in comparison with the variation that occurs with eggs from different individuals. The magnitude of those errors may be obtained by comparing the time for 50 per cent cleavage of two batches of eggs from the same female run at the same time and under the same conditions. For the eggs of *Ciona*, *Dendraster*, and *Urechis* such a comparison gave differences of less than ½ per cent. *Strongylocentrotus* and *Lytechinus* gave differences of less than 1 per cent. The variation that is obtained with eggs of different individuals is contained in the data presented in the following sections.

In order to compare the effects of temperature on the different stages of development it is evidently best to use as far as possible the data for a single batch of eggs. Since, however, it was not always feasible to obtain all of the stages from a single batch of eggs, the results with different batches are also presented.

Ciona intestinalis

The data that have been collected on this form concern mainly the cleavage stages. The later stages require rather detailed investigation to insure comparison of identical stages for embryos raised at different temperatures. Furthermore, this could be done more easily with the sand dollar and the sea urchin.

The data for hatching time was in many cases obtained. As shown above, however, hatching time may not bear a very definite relation to the time of attainment of the various morphological stages. In *Ciona* hatching occurs after the tadpole is fully formed, and it evidently results from the liberation of an enzyme (Berrill, 1929). According to the data presented above (p. 61), hatching occurs earlier in a concentrated than in a dilute culture of embryos, presumably because a higher concentration of the enzyme is present. It is conceivable that even in cultures containing the same concentration of embryos temperature may affect the time of hatching in a manner different from its effect on the time of attainment of the various morphological stages. According to the data presented below, the temperature coefficients obtained for hatching are somewhat different from those for the cleavage stages. On the other hand, the temperature coefficients for the various cleavage stages are very much alike.

The data in Table I give the result of three separate runs, one at 20° and 15°, one at 25° and 15°, and one at 22° and 12°. In the first two columns of each group the time at which 50 per cent of the eggs have reached the indicated stage is given. In the third column are the ratios (Q_5 or Q_{10}) of these values for the lower and higher temperature. In Table II a summary of these ratios for a number of determinations is given. The data are presented for each pair of temperatures separately since, as pointed out in the previous section, each batch of eggs was run at only two different temperatures. The variation in time of development that is obtained with eggs from different individuals may be seen from the following figures for the time of first division in five separate runs at 20.0° C.:

TABLE I

Ciona; times of attainment of various stages for three batches of eggs, each run at two different temperatures; the time for the cleavage stages is given in minutes, that for hatching, in hours; the coefficients, Q_5 and Q_{10} are the ratios of the time at the lower temperature to the time at the higher temperature.

	20.0°	15.0°	Q_5	25.0°	15.0°	Q_{10}	22.0°	12.0°	Q_{10}
2-cell	47.2	77.0	1.63	34.9	77.0	2.21	41.9	118.5	2.83
4-cell	69.0	114.0	1.65	50.5	114.5	2.27	64.0	178.0	2.78
8-cell	91.7	149.0	1.63	66.1	150.5	2.28	78.5	224.5	2.81
16-cell	116.0	195.0	1.68	86.0	194.5	2.26			
Hatching	14.7	25.4	1.73	10.2	25.5	2.50	11.5		

47.2 48.2 47.0 48.3 48.0 Av. = 47.7 a.d. = 0.5

The variation that is obtained at other stages and at other temperatures is of the same relative order of magnitude. The ratios, as shown in Table II, vary less on the whole than do the values for the time of division. In other words, even when the time of development is different in two batches of eggs run at the same two temperatures, the ratios of the time at the different temperatures for each batch tend to be more nearly alike.

It can be seen from Tables I and II that the temperature coefficients for the rate of attainment of the various cleavage stages are alike. We need not present the deviation measures to demonstrate this, since it can be readily seen from Table I that for the same batch of eggs there are no consistent deviations in the coefficients for different stages, and from Table II that for different batches of eggs the range of variation of the average coefficients for the different stages lies within the range of variation of the coefficients obtained for one stage.

TABLE II

Ciona; temperature coefficients; Q_5 and Q_{10} represent the time at the lower temperature divided by the time at the higher temperature.

	Q_5	Q_{10}	Q_{10}
	15.0°/20.0°	15.0°/25.0°	12.0°/22.0°
2-cell	1.63	2.21	2.83
	1.66	2.30	2.91
	1.64	2.24	2.85
	1.66	2.25	2.86
	1.63		
	1.63	2.25	2.86
4-cell	1.65	2.27	2.78
	1.65	2.32	2.81
	1.67	2.29	2.83
	1.63	2.27	2.85
	1.65	2.29	2.84
8-cell	1.63	2.28	2.81
	1.64	2.32	2.85
	1.62		
	1.63	2.30	2.83
16-cell	1.68	2.26	
	1.67	2.25	
	1.63		
	1.66	2.26	
Hatching	1.73	2.50	
	1.76	2.61	
	1.74		
	1.74	2.56	

The temperature coefficients for hatching, however, are apparently greater than for the cleavage stages. At the lowest temperature employed, namely 12°, no hatching was obtained in four separate experiments, although development appeared to be normal in all cases. In three other runs at this temperature cleavage was interfered with. As a possible interpretation of the effect of temperature on hatching, the following might be suggested. Let us assume that hatching depends upon the attainment of a certain concentration of a particular enzyme and that the effect of temperature on the rate of production of the enzyme is the same as its effect on the rate of development. The rate at which the enzyme diffuses away from the embryo as well as its rate of production will then determine its concentration in the membrane. Since the physical process of diffusion would have a lower temperature coefficient than the chemical processes involved in the production of the enzyme, the rate of diffusion would not be slowed up as much, when the temperature is lowered, as the rate of production. This would lead to larger values for the temperature coefficient of hatching as compared with that for development. For the failure to

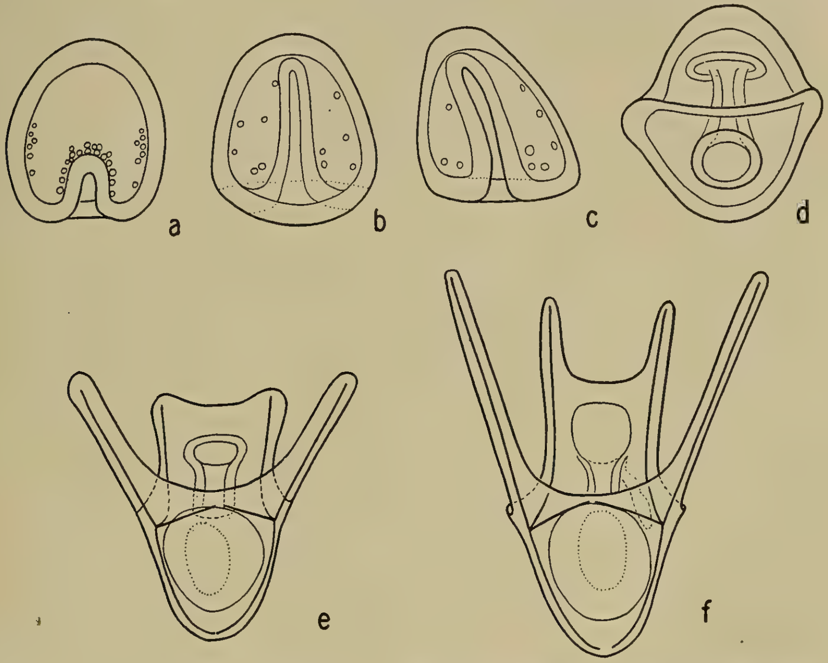

FIG. 1. *Dendraster* embryos. *a*, beginning gastrula; *b*, early prism stage, dorsal view; *c*, early prism stage, side view; *d*, late prism stage, ventral view; *e*, middle pluteus stage; *f*, late pluteus stage.

hatch at a low temperature at which development is normal, we might assume in addition that the enzyme must attain a certain minimum concentration in order to be effective. Considerably more information than has yet been obtained would be necessary to test these points.

Dendraster excentricus

In this form temperature coefficients for gastrulation, prism and pluteus stages as well as for cleavage stages were determined. In a few cases hatching-time was also noted. Some of the stages examined are illustrated in Fig. 1. During gastrulation embryos that are 1/8, 1/4, 1/2, 3/4 or completely gastrulated can be readily distinguished. At the completion of gastrulation the sand-dollar embryo has become flattened on one side (the anterior side in consideration of the direction of swimming of the pluteus), and may at that time be designated as the early prism stage (Fig. 1*b* and *c*). Later, that side of the embryo becomes concave and the archenteric pouches appear. We may designate this stage as the late prism (Fig. 1*d*). Following this the aboral (ventral) arms appear and the gut becomes tripartite, forming the early pluteus. The next stage is just before the appearance of the oral (dorsal) arms, and may be called the middle pluteus stage (Fig. 1*e*). Before this stage the anterior border of the oral lobe is convex and as the oral arms form it becomes concave. This change takes place fairly rapidly and so the time at which this stage is reached can be determined with some accuracy. For the late pluteus stage (Fig. 1*f*) the length of the oral arms serves as a simple criterion in identifying embryos that are comparable.

TABLE III

Dendraster; same description as Table I

	20.0°	15.0°	Q_5	15.0°	10.0°	Q_5	22.0°	12.0°	Q_{10}
2-cell	55.0	89.5	1.63	90.0	184.0	2.04	46.8	135.0	2.88
4-cell	86.5	138.0	1.60	139.5	281.0	2.01	72.0	202.0	2.81
8-cell	109.0	173.0	1.59	174.0	336.0	1.93	98.0	278.0	2.84
16-cell	126.0	203.0	1.61	—	—	—	—	—	—
Hatching	7.6	11.8	1.55	—	—	—	7.0	17.4	2.49
1/8 gastrulated	12.7	20.9	1.65	20.2	39.8	1.97	10.9	30.4	2.79
1/4 gastrulated	13.2	21.5	1.63	—	—	—	—	—	—
1/2 gastrulated	14.2	22.9	1.61	23.1	46.0	1.99	—	—	—
3/4 gastrulated	15.0	24.1	1.61	24.6	49.2	2.00	12.6	36.0	2.82
Early prism	15.9	26.4	1.66	26.3	53.0	2.01	—	—	—
Late prism	20.8	33.1	1.59	—	—	—	—	—	—
Early pluteus	23.5	38.6	1.64	38.0	77.5	2.04	—	—	—
Middle pluteus	33.1	54.6	1.65	55.0	113.5	2.06	—	—	—
Late pluteus	—	—	—	—	—	—	48.7	142.0	2.92

Table III gives the data for single batches of eggs, one run at

20.0° and 15.0°, one at 15.0° and 10.0° and one at 22.0° and 12.0°. The ratios of the time at the lower temperature to that at the higher temperature are given in the third, sixth and ninth columns of the table (Q_5 and Q_{10}). In Table IV the values of these ratios for five pairs of temperatures are given. Each value is, of course, obtained

TABLE IV

Dendraster; same description as Table II

	15.0°/20.0°		10.0°/15.0°	12.0°/22.0°		15.0°/25.0°	8.0°/18.0°
2-cell stage	1.63		2.04	2.88		2.21	3.91
	1.64	1.61	1.95	2.78		2.18	3.85
	1.59	1.61	2.00	2.79		2.25	
		1.63	2.06	2.82			
		1.62	2.01	2.82		2.21	3.88
4, 8 and 16-cell stage	1.60		2.01	2.81		2.26	3.82
	1.62		1.92	2.78	2.75	2.22	
	1.58		1.94	2.80	2.85		
	1.59				2.78		
	1.60		1.96		2.80	2.24	3.82
Gastrulation	1.65		1.97	2.79		2.17	3.83
	1.63	1.66	1.99	2.82		2.22	3.94
	1.61	1.62	2.00	2.79			
	1.61	1.61	1.93	2.76			
	1.58	1.59	2.00				
	1.59	1.60	2.00				
			2.03				
		1.60	1.99	2.79		2.20	3.98
Prism	1.66		2.01	2.79		2.17	3.90
	1.59		2.08				3.82
			2.02				
	1.63		2.04	2.79		2.17	3.86
Pluteus	1.64		2.04	2.85			3.86
	1.65		2.06	2.92			4.01
			2.03	2.76			3.95
			2.00	2.82			
	1.65		2.03	2.84			3.94

from a single batch of eggs run at the two temperatures. In some cases values for a number of stages (as is the case in Table I) were obtained from a single batch of eggs. It can be readily seen from the tables that the temperature coefficients for the various cleavage stages are the same. Furthermore, the coefficients for the later stages fall

TABLE V

Urechis; same description as Table I.

	20.0°	15.0°	Q_5	22.0°	12.0°	Q_{10}
2-cell......	74.5	120.0	1.61	65.0	180.5	2.78
4-cell......	104.0	162.0	1.56	88.0	249.0	2.83
8-cell......	129.0	206.5	1.60	113.5	319.0	2.81
16-cell......	158.5	254.5	1.61	39.5	385.0	2.76

within the range of values obtained for the cleavage stages. The range of temperatures (8.0° to 25.0°) covered is practically that outside of which normal development can no longer be obtained.

If the figures obtained for *Dendraster* are compared with those for *Ciona* it may be seen that they are very much alike. The average values for all the cleavage stages are given in Table XIII along with those of the other forms investigated. This similarity in the coefficients for *Dendraster* and *Ciona* may best be discussed after considering the other forms.

Urechis caupo

Only the data for the cleavage stages of *Urechis* eggs are presented here, inasmuch as they present the same difficulties as *Ciona* for obtaining reproducible results in the later stages. The results given in Tables V and VI show that for the eggs of this form too the temperature

TABLE VI

Urechis; same description as Table II.

	15.0°/20.0°	10.0°/15.0°	12.0°/22.0°
2-cell	1.61	1.95	2.78
	1.59	2.08	2.76
	1.58	2.15	2.84
	1.60		
	1.64		
	1.63		
	1.61	2.06	2.79
4, 8 and 16-cell	1.56	1.97	2.83
	1.62	2.04	2.79
	1.65	2.11	2.81
	1.60		2.71
	1.63		2.78
	1.59		2.76
	1.61		2.82
	1.61	2.04	2.79

coefficients of the rate of attainment of the various cleavage stages are alike. It may also be readily seen that the values obtained with *Urechis* eggs are quite similar to those obtained with eggs of *Dendraster* and *Ciona* at the same temperatures.

Strongylocentrotus purpuratus

Gastrulation and prism stages as well as cleavage stages of the *Strongylocentrotus* eggs were examined. The extent of the gut cavity rather than the extent of invaginated cells was used as a criterion for determining the stage of gastrulation. This was done because the primary mesenchyme that covers and extends forward from the gut does not change very markedly in appearance as gastrulation proceeds. Although the results with *Strongylocentrotus* vary more than with the forms previously presented, it may be readily seen from Tables VII and VIII that no consistent differences occur in the

TABLE VII

Strongylocentrotus; same description as Table I.

	20.0°	15.0°	Q_5	20.0°	7.5°	$Q_{12.5}$
2-cell	77.0	105.0	1.36	76.3	227.0	2.98
4-cell	126.0	174.0	1.38	125.0	383.0	3.06
1/8 gastrulated	25.0	32.7	1.31	24.7	73.5	2.98
1/2 gastrulated	28.8	37.0	1.29	28.5	89.0	3.12
3/4 gastrulated	31.4	42.0	1.34	—	—	—
Prism with skeletal rods	49.0	59.0	1.34	48.0	140.0	2.92

TABLE VIII

Strongylocentrotus; same description as Table II.

	Q_5 15.0°/20.0°	$Q_{12.5}$ 7.5°/20.0°
2- and 4-cell	1.36	2.98
	1.30	2.89
	1.31	2.97
	1.38	3.08
	1.30	3.06
	1.33	3.00
Gastrulation	1.31	2.98
	1.37	3.04
	1.29	3.12
	1.39	3.09
	1.37	
	1.35	3.06

TABLE IX

Lytechinus; same description as Table I.

	15.0°	10.0°	Q_5
2-cell	137.0	281.0	2.05
1/8 gastrulated	30.2	63.5	2.08
1/2 gastrulated	35.0	73.0	2.09
Early prism	58.5	116.5	1.99
Late prism	73.0	151.0	2.07

temperature coefficients of the cleavage and the later stages. The average values of the temperature coefficients for *Strongylocentrotus* differ considerably from those for *Ciona*, *Dendraster* and *Urechis* at the same temperatures, as may be seen in Table XIII.

TABLE X

Lytechinus; same description as Table II.

	Q_5 10.0°/15.0°
Cleavage	2.05
	1.91
	1.87
	1.94
Gastrulation	2.08
	2.04
	2.09
	2.07
Prism	1.99
	2.10
	2.07
	2.05

Lytechinus anamesus

The time of attainment of the two-cell stage, gastrulation and prism stages was determined for the eggs of this sea urchin at only one pair of temperatures. The results are presented in Tables IX and X. The data are less extensive than for the other forms; nevertheless it can be seen that the temperature coefficients for the later stages are very much like those of the two-cell stage. A comparison of the average value with those of the other forms is given in Table XIII.

Number of Cells in Sea-urchin Gastrulæ Raised at Different Temperatures

From the similarity of the temperature coefficients of the gastrula and the early cleavage stages, it is to be expected that the number of cells present in gastrulæ raised at different temperatures should be the same, provided, of course, that the later cleavage stages have the same temperature coefficient as the earlier ones. Since the data in the early literature (see above) showed different numbers of cells present in embryos raised at different temperatures, this point was reinvestigated.

Two methods were used in estimating the cell number, that of Marcus (1906) and that of Koehler (1912). Marcus' method consisted in counting the number of nuclei (n) in an optical section at a great circle. Then the total number of cells (N) is given by

$$N\left(\frac{2\pi r}{n}\right)^2 = 4\pi r^2,$$

where r is the radius of the embryo. It is assumed that the surface outline of each cell is a square. This equation reduces to

$$N = \frac{n^2}{\pi}$$

and it is unnecessary to measure the radius as Marcus did. Koehler counted the number of nuclei in a small area of the surface. The number of nuclei per unit area (n/s^2) multiplied by the surface area of the embryo gives the total number of cells

$$N = 4\pi r^2 \cdot \frac{n}{s^2},$$

where the radius, r, is measured to the middle of the wall. It would be better, however, to measure the radius to a point one-third the distance between the inner and outer surfaces of the wall, since the centers of the nuclei correspond to about that position.

For embryos that have gastrulated corrections must, of course, be made for the invaginated cells, if the total cell number is desired. The measurements presented here were made on embryos that had just begun gastrulation. The number of mesenchyme cells could be estimated roughly and the small invagination could be considered to occupy the same area as if the cells were still on the spherical surface. The surface area was calculated from the formula for an oblate spheroid. Neither of the two methods gave very accurate results. Koehler claims that his method is better since one is not troubled with having the optical section coincide with the greatest circle and with higher and

TABLE XI

Strongylocentrotus; number of cells in beginning gastrulæ.

A		*B*	
20.0°	7.5°	20.0°	7.5°
680	680	776	688
769	799	780	815
623	680	705	863
651	738	690	752
799	708	802	885
799	644	801	829
769	597	854	780
680	608	806	809
721	682	777	803

lower nuclei that might or might not be in the great circle. But in Koehler's method the unit square of surface in which the nuclei are counted is actually a spherical surface and if a large area is taken, then too many nuclei are counted, whereas if a small area is taken the error due to the curvature is negligible but the counting becomes more difficult because a considerable fraction of the nuclei are cut by the boundaries of the area.

In Table XI the cell numbers are given for eight beginning gastrulæ raised at 20.0° and eight of the same batch raised at 7.5° C. The counts were made on slides of preserved embryos, only those in side view being chosen. The first two columns (*A*) were obtained from counts of the nuclei in optical section. The second two columns (*B*) are from counts of nuclei in a unit area of surface. Figures 2*a* and *b* illustrate the nuclei in optical section and Figs. 2*a′* and *b′* the surface nuclei of a 20.0° and a 7.5° embryo. These two embryos happened to show the same number of nuclei (45) in optical section and gave a total (including 35 mesenchyme cells) of 680 cells. The cell numbers from the counts of the surface nuclei are 776 and 688 respectively. The other values presented in the table vary considerably, yet there are no consistent differences between the warm and cold series.

DISCUSSION

Numerous methods have been employed by biologists to express the relation between temperature and the rate of biological processes. An extensive review is given by Bělehrádek (1935). Although we are not especially concerned here with establishing any particular relation, it is of some interest to examine how well two of the commonly employed formulæ fit our data. We shall consider first the Arrhenius equation which has been extensively applied by Crozier and his collaborators (1924–) to various biological processes including the rate of development. If the logarithm of the velocity of the particular process is plotted against the reciprocal of the absolute temperature, then, according to the Arrhenius equation, a straight line should be expected. In the graph of Fig. 3 the data for the first cleavage of *Ciona*, *Dendraster* and *Urechis* are so plotted. Only the values for the first division were used since these were the most reliable ones obtained. The figures from which the graph was obtained are given in Table XII. These figures do not represent averages of all the first cleavage values but were taken from cases where the cleavage time obtained at one temperature was practically identical in two separate runs in which the temperature of the second bath had been changed. This was done in order to cut down the variability that would be obtained with different batches of eggs and, of course, because only two temperature baths were available at one time. The graphs of Fig. 3 are certainly not straight lines. Small portions of these graphs might be considered as such. But in order to conclude that the

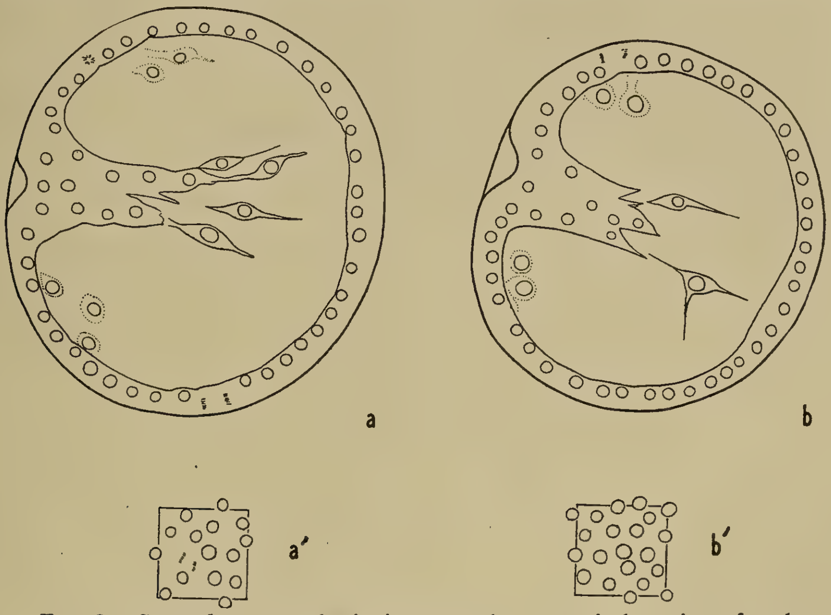

FIG. 2. *Strongylocentrotus* beginning gastrulæ: *a*, optical section of embryo raised at 20.0° C.; *a′*, nuclei in unit area of surface of same embryo; *b*, optical section of embryo raised at 7.5° C.; *b′*, nuclei in unit area of surface of same embryo.

TABLE XII

Time for first cleavage.

	Ciona	Dendraster	Urechis
8.0° C.		248.0	
10.0		184.0	262.0
12.0	117.9	135.0	183.5
15.0	77.0	89.5	120.0
18.0	56.5	63.5	89.5
20.0	47.7	54.75	74.5
22.0	42.2	46.75	64.5
25.0	35.1	40.5	

Arrhenius equation applies we would need to make the now prevalent but as yet unproved assumption of different limiting reactions of a catenary series at different temperature intervals. If such an assumption were to be made, it would be desirable to have considerably more points on the graph.

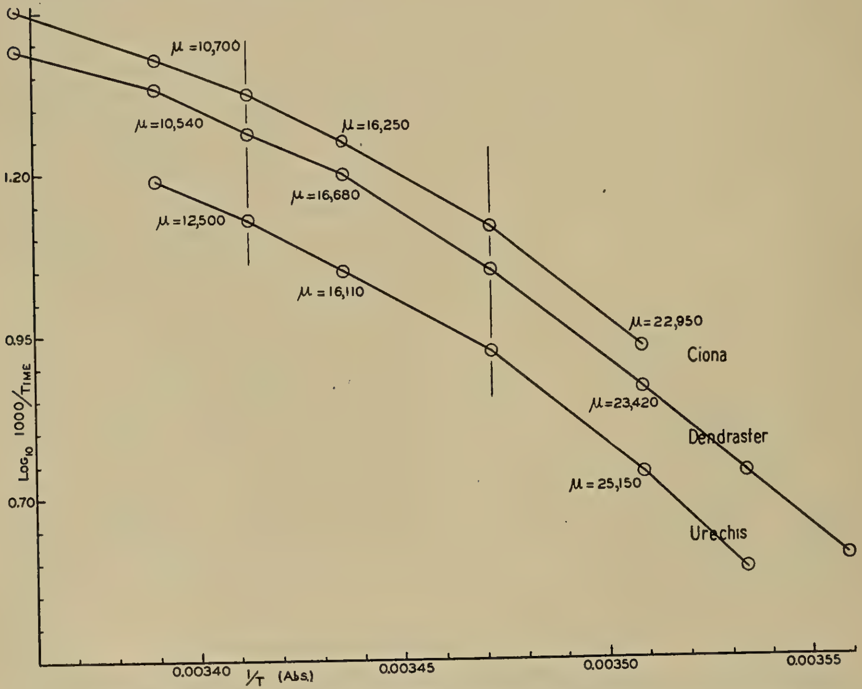

FIG. 3. First cleavage data of Table XII plotted as logarithm of the rate against the absolute temperature.

There is a rather simple relation between temperature and the rates of biological processes that was first applied to developing eggs by Krogh (1914). This relation is simply that the rate is directly proportional to the temperature. Support for this view has been obtained by a number of investigators, and recently Ephrussi (1933) has shown that the effect of temperature on the rate of cleavage of sea-urchin eggs is fairly well expressed by it. In Fig. 4 the data of Table XII are plotted as the reciprocal of the time for first cleavage against the temperature. The points fall fairly well on a straight line for most of the temperature range, as they should if the rate is directly proportional to the temperature. At the lower temperatures deviations occur in the graphs for *Dendraster* and *Urechis*. The graph for *Ciona*, the data for which are more reliable than in the case of the other forms, is a very good straight line.

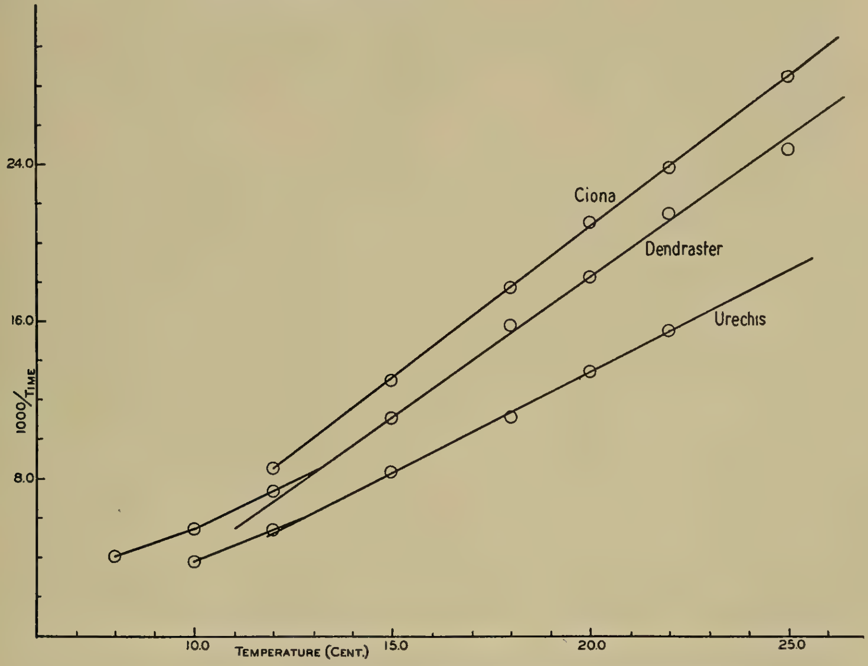

FIG. 4. First cleavage data of Table XII plotted as rate against centigrade temperature.

The significance of this linear relation between the rate of a biological process and temperature is quite obscure. Bělehrádek employs a modified equation which may be written

$$v = a\,(t - \alpha)^b$$

in which v is the velocity, t the temperature and a, α and b constants. When the exponent $b = 1$, this becomes the linear relation. It is assumed that the temperature coefficients give a measure of the relative viscosity although just how the constants of the formula are related to viscosity is not clear. Support of this view is given by Bělehrádek and Mládek (1934), who found higher values for the temperature coefficients of respiration of more viscous meal-worm larvæ than of less viscous ones. Bodine and Thompson (1935) showed, however, that this is not necessarily the case, inasmuch as dehydrated grasshopper embryos gave the same values for the temperature coefficient of respiration as normal embryos.

If the temperature coefficients for the five forms investigated are compared, it is seen that four of them, *Ciona*, *Dendraster*, *Urechis* and *Lytechinus*, are very similar, whereas *Strongylocentrotus* gives values that are quite different. In Table XIII the mean values for the cleavage stages are presented. An examination of the values for the temperatures 15° and 20° (first column of table) shows that the coefficient for *Strongylocentrotus* is much lower than for the others. At the temperatures 7.5° and 20° the figure for *Strongylocentrotus* in this table is 3.0, whereas the value for *Dendraster* in the temperature interval 8° and 18° is 3.9 (Table IV) and the extrapolated value for 7.5° and 20° is 4.7.

TABLE XIII

Temperature coefficients for cleavage. (Mean values and their standard deviations taken for all the cleavage data.)

	Q_5	Q_{10}	Q_5	Q_{10}	$Q_{12.5}$
	15°/20°	15°/25°	10°/15°	12°/22°	7.5°/20°
Ciona	1.65±0.018	2.27±0.033	———	2.84±0.035	———
Dendraster	1.61±0.02	2.22±0.032	1.99±0.053	2.80±0.038	4.7*
Urechis	1.61±0.026	———	2.05±0.079	2.79±0.029	———
Lytechinus	———	———	1.94	———	———
Strongylocentrotus	1.33±0.037	———	———	———	3.00±0.076

* Calculated value.

In view of the striking difference shown by *Strongylocentrotus*, it is hardly worthwhile at present to try to account for the similarity shown by the others. The closeness of taxonomic relationship evidently has no bearing on the results, inasmuch as the two other echinoderms, *Dendraster* and *Lytechinus*, differ from *Strongylocentrotus*, whereas they are like *Ciona* and *Urechis*. It also appears that the eggs of one species may have different temperature coefficients, as Ephrussi (1933) has shown for the results of Horstadius (1925) on the rate of cleavage of *Paracentrotus lividus* eggs in summer and in winter. This particular case is taken by Ephrussi to illustrate the rule of Skrabal (1916) that fast reactions have lower temperature coefficients than slow reactions. Zawadowsky and Sidorov (1928) present values for the cleavage of sea urchins (three species), frog and *Ascaris* eggs that are in agreement with that rule. The data presented in this paper, however, do not fit into that scheme even in the case of the three echinoderms alone. For example, *Strongylocentrotus* has a slower rate of cleavage than the other two echinoderms and also a lower temperature coefficient.

It is the relation between the temperature coefficients for cleavage and for organogenesis that is of particular interest here. That the various early cleavage stages have the same temperature coefficients is perhaps to be expected since the early cleavage stages represent the same sort of process. This statement must be qualified, however, for development up to the first cleavage since in addition to the kind of events occurring in the later cleavages there is also fertilization, fusion of pronuclei, extrusion of polar bodies, etc. to be considered. The temperature coefficients for the various phases of the first cleavage have been investigated by Ephrussi (1927) and for fertilization alone by Chase (1935). Different values are found by Ephrussi for various stages from fertilization to first division in *Paracentrotus*, but this would not prevent the overall values for the various cleavage stages (including the first) from being the same. Fry (1936) finds, on the other hand, the same temperature coefficient for the mitotic phases as for cleavage in *Arbacia*. The kinds of changes that occur in the later stages of differentiation (i.e. from gastrulation on) are manifestly different from the cleavage stages. The interpretation that may be made of the identity of temperature coefficients throughout development depends, of course, on the particular meaning assigned to temperature coefficients in general. But this involves some controversial questions that are beyond the scope of this paper.

Our results show that we cannot within the range of temperatures used dissociate cleavage and organogenesis by means of temperature. It would be of interest to know whether other agents or extremes of temperature might effect such a separation so that the rates may be affected differently or that a process such as gastrulation may be made to proceed without the accompanying cell division.

SUMMARY

The effect of temperature on the rate of development of eggs of *Ciona intestinalis*, *Dendraster excentricus*, *Urechis caupo*, *Strongylocentrotus purpuratus* and *Lytechinus anamesus* has been investigated. It is found that not only do the various early cleavage stages have the same temperature coefficient, but the values for the later stages of differentiation are the same as for cleavage.

In *Ciona*, the coefficient for hatching time is different from that foı cleavage. An interpretation of this based on the production and diffusion of a hatching enzyme is suggested.

Counts of the number of cells of gastrulæ of *Strongylocentrotus* show no significant differences in embryos raised at different temperatures.

The data are fairly well expressed by the assumption of a linear relation between the rate of development and temperature.

The values of the coefficients for all the forms investigated, with the exception of *Strongylocentrotus*, are very closely alike, although the rates of development are quite different. *Strongylocentrotus* eggs, which develop more slowly than *Dendraster* or *Lytechinus*, give lower values of the temperature coefficient.

LITERATURE CITED

ATLAS, M., 1935. The effect of temperature on the development of Rana pipiens. *Physiol. Zoöl.*, **8**: 290.

BĚLEHRÁDEK, J., 1935. Temperature and living matter. *Protoplasma Monographien*, vol. 8.

BĚLEHRÁDEK, J., AND J. MLÁDEK, 1934. Richesse en eau du bioplasme et la grandeur du coefficient thermique des oxydations. *Protoplasma*, **21**: 335.

BERRILL, N. J., 1929. Studies in tunicate development, Part 1. General physiology of development of simple ascidians. *Phil. Trans. Roy. Soc. London*, B, **218**: 37.

BIERENS DE HAAN, J. A., 1913. Über homogene und heterogene Keimverschmelzungen bei Echiniden. *Arch. Entw.-mech.*, **36**: 474.

BODINE, J. H., AND V. THOMPSON, 1935. Temperature coefficients and viscosity. *Jour. Cell and Comp. Physiol.*, **6**: 255.

BORING, A. M., 1909. On the effect of different temperatures on the size of the nuclei in the embryo of Ascaris megalocephala, with remarks on the size-relation of the nuclei in univalens and bivalens. *Arch. Entw.-mech.*, **28**: 118.

CHASE, H. Y., 1935. The effect of temperature on the rate of the fertilization reaction in various marine ova. *Biol. Bull.*, **69**: 415.

CROZIER, W. J., 1924. On the possibility of identifying chemical processes in living matter. *Proc. Nat. Acad. Sci.*, **10**: 461.

CROZIER, W. J., 1924. On biological oxidations as function of temperature. *Jour. Gen. Physiol.*, **7**: 189.

CROZIER, W. J., 1926. The distribution of temperature characteristics for biological processes; critical increments for heart rates. *Jour. Gen. Physiol.*, **9**: 531.

DRIESCH, H., 1900. Studien über das Regulationsvermögen der Organismen 4. Die Verschmelzung der Individualität bei Echinidenkeimen. *Arch. Entw.-mech.*, **10**: 411.

EPHRUSSI, B., 1927. Sur les coefficients de température des différentes phases de la mitose des oeufs d'Oursin (Paracentrotus lividus, lk.) et de l'Ascaris megalocephala. *Protoplasma*, **1**: 105.

EPHRUSSI, B., 1933. Contribution à l'analyse des premiers stades du développement de l'oeuf. Action de la température. *Arch. de Biol.*, **44**: 1.

ERDMANN, RH., 1908*a*. Experimentelle Untersuchung der Massenverhältnisse von Plasma, Kern und Chromosomen in dem sich entwickelnden Seeigelei. *Arch. f. Zellforsch.*, **2**: 76.

ERDMANN, RH., 1908*b*. Kern- und Protoplasmawachstum in ihren Beziehungen zueinander. *Erg. Anat. und Entw.-gesch.*, **18**: 844.

FRY, H. J., 1936. Studies of the mitotic figure. V. The time schedule of mitotic changes in developing Arbacia eggs. *Biol. Bull.*, **70**: 89.

GODLEWSKI, JUN. E., 1908. Plasma und Kernsubstanz in der normalen und der durch äussere Faktoren veränderten Entwicklung der Echiniden. *Arch. Entw.-mech.*, **26**: 278.

GRAY, J., 1928. The growth of fish. III. The effect of temperature on the development of the eggs of Salmo fario. *Brit. Jour. Exper. Biol.*, **6**: 125.

HERTWIG, O., 1898. Ueber den Einfluss der Temperatur auf die Entwicklung von Rana fusca und Rana esculenta. *Arch. f. Mikrosk. Anat.*, **51**: 319.

HÖRSTADIUS, S., 1925. Temperaturanpassung bei den Eiern von Paracentrotus lividus Lk. *Biologia Generalis*, **1**: 522.

KOEHLER, O., 1912. Über die Abhangigkeit der Kernplasmarelation von der Temperatur und vom Reifezustand der Eier. *Arch. f. Zellforsch.*, **8**: 272.

KROGH, A., 1914. On the influence of the temperature on the rate of embryonic development. *Zeitschr. f. allg. Physiol.*, **16**: 163.

MARCUS, H., 1906. Über die wirkung der Temperatur auf die Furchung bei Seeigeleiern. *Arch. Entw.-mech.*, **22**: 445.

MORGAN, T. H., 1901. The proportionate development of partial embryos. *Arch. Entw.-mech.*, **13**: 416.

MORGAN, T. H., 1903. The gastrulation of the partial embryos of Sphærechinus. *Arch. Entw.-mech.*, **16**: 117.

PETER, K., 1905. Der Grad der Beschleunigung tierischer Entwicklung durch erhöhte Temperatur. *Arch. Entw.-mech.*, **20**: 130.

SKRABAL, A., 1916. Cited by Ephrussi, 1933.

TYLER, A., 1933. On the energetics of differentiation. A comparison of the oxygen consumption of "half" and whole embryos of the sea-urchin. *Publ. Staz. Zool. Napoli*, **13**: 155.

TYLER, A., 1935. On the energetics of differentiation. II. A comparison of the rates of development of giant and of normal sea-urchin embryos. *Biol. Bull.*, **68**: 451.

ZAWADOWSKY, M. M., AND C. M. SIDOROV, 1928. Die Abhängigkeit der Entwicklung der Eier von Ascaris megalocephala, Ascaris suilla und Toxascaris limbata von der Temperatur. *Arch. Entw.-mech.*, **113**: 536.

ON THE ENERGETICS OF DIFFERENTIATION. IV

COMPARISON OF THE RATES OF OXYGEN CONSUMPTION AND OF DEVELOPMENT AT DIFFERENT TEMPERATURES OF EGGS OF SOME MARINE ANIMALS

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The results of these experiments show principally that, for the eggs of certain marine animals, the total oxygen consumed in reaching the same stage of development at different temperatures is the same.

THEORETICAL PART

It has been assumed (Tyler, 1933, 1935) that energy is required for the differentiation as well as the maintenance and growth processes of development and that this energy is supplied by reactions involving oxygen.

The terms maintenance, growth and differentiation have been used in various ways by different investigators. By maintenance I mean the processes by which the egg or embryo is kept alive in a "resting condition." An unfertilized egg illustrates this. Also a fertilized egg in which development has been reversibly stopped would illustrate maintenance. But it cannot be assumed that the maintenance energy requirement of a developing egg is equivalent to the metabolism of an unfertilized or of a fertilized egg in which development has been stopped, for the maintenance requirement of a fertilized egg is probably different from that of an unfertilized egg; it very likely changes throughout development and under different conditions. In other words, it takes different amounts of energy to keep different kinds of cells alive under the same conditions, and the same kind of cell alive under different conditions.

By growth we mean the processes by which food material is converted into protoplasm. This may not be the most convenient way in which to define this term in the case of the early development of a marine egg. It is, however, more consistent with the generally employed definitions of growth. The difficulties arise in distinguishing between the food materials and the protoplasm of an egg. We recognize, however, certain constituents such as the yolk, fat, and some pigments as essentially non-protoplasmic materials. In the case of most small eggs these materials are distributed throughout the cytoplasm. In the case of large eggs, such as those of birds, a region which is preponderantly protoplasmic may be distinguished from one which is chiefly yolk. The embryo arises in the protoplasmic area and evidently contains a negligible amount of food material. Thus in studies on the growth of bird or fish embryos it is essentially the conversion of food materials into protoplasm that is being measured. In the case of unicellular organisms growth studies likewise give a measure of the conversion of food materials of the medium into protoplasm of the cells. For small eggs it has been the custom either to consider growth as not occurring until actual increase in mass by absorption of materials from the outside takes place, or to use the term growth as loosely synonymous with development. It is evident, however, that there is growth in small eggs during the period before actual increase in mass takes place. The difference is that the nutritive material is intracellular.

Distinction should also be made between growth and what we may call storage. The increase in size of the young oöcyte as it is transformed into the ripe egg may be considered as illustrating storage since it is principally food materials that accumulate. It may seem that this manner of regarding growth leads to a paradox; we have cases of no increase in mass in which we do not consider growth to be occurring. But the ordinary definition of growth as increase in mass is meaningless unless the system is specified. Our definition considers the protoplasm to be the growing system. We define growth in this manner in order to be able when necessary to utilize the general results of growth studies on various kinds of living things.

Differentiation may be regarded as the changes in form that occur during development, the so-called morphogenetic and histogenetic changes. Strictly speaking, this is one aspect of what may be called differentiation. The production of the typical chemical substances characteristic of the various tissues must also be included. However, this also fits into our definition of growth. There is no point at present in attempting to classify it specifically with growth or with the changes in form. It rests, of course, with the actual experimental evidence to decide what the exact relationships are.

The relation of cleavage to differentiation has been considered in the previous paper. In the early experiments of Lillie (1906), a sort of differentiation without cleavage has been described. But it is questionable whether in this case typical differentiation processes have taken place. This is not merely a matter of the use of words, for if the kinds of changes described by Lillie are comparable with those undergone in normal development, such embryos would furnish important material for the analysis of the form changes and histochemical changes. It has, of course, been long known that cleavage and growth may go on without differentiation (e.g., anidian chick embryos, Dareste, 1882, Grodzinski, 1933). But that should not lead us to expect the reverse to be true.

In distinguishing between maintenance, growth and differentiation we do not mean to imply that these are independent processes in development. They are undoubtedly interrelated, but we can effect a separation conceptually and to some extent experimentally. An excellent review of normally occurring and experimentally produced cases of dissociation of the various embryological processes is given by Needham (1933), and justification for the general point of view presented here is contained therein.

Concerning the effect of temperature on the rate of respiration and on the rate of development, the different processes involved should be considered.

If as the temperature is lowered the requirements for these processes are decreased in the same proportion, we should expect the decrease in rate of development to be the same as the decrease in rate of oxygen consumption. This would not tell us much about maintenance, growth and differentiation, but it would mean that the efficiency with which the available energy is utilized remains the same at different temperatures. If, however, the requirements for the various processes changed in different ways with change in temperature we should not expect such a simple relation. Unfortunately we cannot state *a priori* in what manner the various processes should be affected by temperature. But it is possible to get at this experimentally. Thus it should be possible to learn the manner in which maintenance is affected by studying the effect of temperature on the metabolism and length of life of unfertilized eggs. The duration of the fertilizable condition must also be investigated, since strictly speaking we must regard maintenance as not merely the process of keeping the cells alive but also of keeping them in a condition in which they are capable of functioning normally. Such an investigation involves certain difficulties which remain to be overcome before the results can be presented.

This work concerns only the effect of temperature on the rate of development and rate of oxygen consumption. Several such investigations have been made in the past. The results of Loeb and Wasteneys (1911) are the most often cited and generally accepted. They found the temperature coefficient of the rate of development to be larger than that of the rate of oxygen consumption. At low temperatures, then, the total oxygen consumed in reaching a given stage of development would be greater than at higher temperatures. Loeb and Wasteneys worked with sea-urchin eggs and considered only the early cleavage stages. But as we have seen in the previous papers, the temperature coefficients for the cleavage and the later stages are the same. They determined their temperature coefficients for respiration by running a batch of eggs for some time at one temperature and then changing the temperature on the same batch of eggs. Thus, the eggs are not in the same stage of development and, as Ephrussi (1933, p. 115) points out, the results are not strictly comparable, since as development proceeds the rate of oxygen consumption rises.

Ephrussi, working also on sea-urchin eggs, gets different results. Using the time from the two-cell stage to hatching as the criterion for rate of development, he finds that the total oxygen consumption is the same at different temperatures, in the interval from 10° to 23°. Above 23° the total oxygen consumption is greater than at the lower temperatures, but Ephrussi states that it is not certain that development would proceed very far at these higher temperatures. When, however, he takes the interval from fertilization to the two-cell stage, he finds the total oxygen consumption to be the same only in the interval between 16° and 23°. Above 23° the total oxygen consumption is higher and below 16° it is lower than at the other temperatures. He supposes that these differences only occur in the early stages and are obscured in the longer time interval and increasingly greater oxygen consumption up to hatching.

Measurements of the rate of cleavage and rate of oxygen consumption at different temperatures have been made by Fauré-Fremiet (1924) on eggs of *Sabellaria*. From his results it appears that the total oxygen consumption in reaching the same stage of development is different at different temperatures.

The same sort of experiment has been performed on eggs of the frog by Barthélemy and Bonnet (1926), using the bomb calorimeter. They found that between 8° and 21° there was the same expenditure of energy in reaching the stage at which the external gills disappear.

In insects, Krogh (1914) and more recently Crescitelli (1935) have determined the rate of pupal development and of respiration at different temperatures. They obtained conflicting results. Krogh used pupæ of the beetle, *Tenebrio,* and found that in the interval of 20° to 33° the total CO_2 produced during pupal life was the same at different temperatures. Crescitelli used pupæ of the moth, *Galleria,* and found in the interval of 20° to 40° the total O_2 consumed or CO_2 produced to be different at different temperatures. He gets a minimum total gas exchange at 30° C. with regular increases to each side of that temperature. Dobzhansky and Poulson (1935) present some data on pupæ of *Drosophila pseudoöbscura* from which it appears that the total oxygen consumption at 25° and 14° is of the same order of magnitude. Similar results are obtained by Brown (1928) with *Ascaris* eggs and by McCoy (1930) with hookworm eggs.

If in the case of the sea-urchin egg the total oxygen consumed at the lower temperatures were much greater than at the higher temperatures, as the results of Loeb and Wasteneys show, we should expect a rather interesting result. The sea-urchin embryo dies within a limited time after entering the pluteus stage unless food is supplied. If, then, we allow sea-urchin eggs to develop at different temperatures on their own initial food supply, the retardation in time of death will not be the same as the retardation in rate of development but will be the same as the retardation in rate of oxygen consumption, since that measures the rate at which the original food supply is being depleted. For example, if we take Loeb and Wasteneys' figures of 7.3 and 2.0 for the coefficients of rate of cleavage and rate of oxygen consumption for 7°–17°, and assuming (as they do) that the values for cleavage hold throughout development, then development will be slowed up 7.3 times at 7° as compared with 17° but the length of life will be prolonged only 2.0 times. Thus, even allowing for the short time in which the pluteus remains alive without undergoing any developmental changes, we should not expect the embryos raised at 7° to have developed as far at the time of death as those at 17°. In fact, it appears from the figures that the embryos raised at 7° would be expected to die in the late prism stage. Thus below a certain temperature embryos may develop normally but would die before being able to feed, since in the prism stage the mouth is not open. We have investigated this particular point and have found no such precocious dying at the lower temperatures. As is evident in the previous paper, embryos raised at the lower temperatures develop as far as those raised at the higher temperatures. While this sort of evidence shows that enormous differences in the temperature coefficients of development and respiration do not occur with our material, it might well be that smaller differences exist. Our experimental evidence, however, gives no significant differences.

EXPERIMENTAL PART

Material and Methods

The eggs of four marine animals were used; namely, the sand-dollar, *Dendraster excentricus,* the sea-urchin, *Strongylocentrotus purpuratus,* the ascidian, *Ciona intestinalis,* and the gephyrean worm, *Urechis caupo.*

The method of determining the rate of development at different temperatures is given in the previous paper. One or more determinations of the temperature coefficient of the rate of development were made on each set of eggs used for the respiration experiments. For this purpose eggs from the same fertilized lot that were used for oxygen consumption measurements were placed in the temperature baths at the same time as the manometers. These egg suspensions were of about the same concentration as the suspensions in the manometer vessels and were subjected to the same shaking. The eggs in the manometer vessels, used for the respiration measurements, were also examined at the end of each run to check their condition as well as the stage of development.

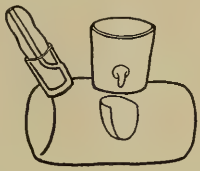

Fig. 1. Type of manometer vessel used in these experiments.

The respiration was measured by means of the Warburg manometer. The vessels used are of the type illustrated in Fig. 1. They are quite small, the calibration volumes ranging from 2.8 to 3.0 cc., which makes them particularly suitable in cases in which only small quantities of material can be obtained. The manometer capillaries were also small (about 0.6 mm. diameter) and the T connection for the arm that hangs into the bath was made much smaller than usual, thus reducing the gas volume outside of the bath. These vessels have been checked against the standard conical type. The amount of fluid with which the vessels can be filled depends upon the rate of shaking. As much as 1.5 cc. of fluid can be used with moderate shaking (about twenty round-trip shakes per minute). In most of the experiments, however, the vessels were filled with 1.1 cc. of fluid, which gives vessel constants for oxygen at 20° ranging around 0.18. As has often been pointed out (see especially Whitaker, 1933), the rate of shaking is of considerable importance when measurements on marine eggs are being made, for one is concerned not only with insuring adequate diffusion of oxygen but also with avoidance of injury to the material. We have determined the optimum shaking for our material and find slow rates of 40 to 70 round-trip shakes per minute at 3.5 cm. amplitude to be satisfactory for dilute egg suspensions (up to 0.5 mg. of nitrogen in 1.1 cc., which for *Strongylocentrotus* corresponds to a volume concentration of 1 : 100). For more concentrated suspensions stronger shaking is necessary, not only because of the greater oxygen consumption, but also because at the same rate of shaking the contents are less disturbed when a concentrated egg suspension is employed. For suspensions giving 2.5 mgm. nitrogen in 1.1 cc. the shaking was 70 to 100 round-trips at 4.5 cm. amplitude.

The quantity of eggs employed was determined after each run by measuring the nitrogen content by a micro-Kjeldahl method. Since this determination could be made to 0.01 mgm. N, the error involved when the quantity of eggs corresponds to 1.0 mgm. N is less than 1 per cent. In some instances the actual number of eggs used was determined by counting.

With the higher ratio of fluid to gas volumes obtained in our vessels the solubility coefficient of O_2 (Bunsen's coefficient, α) is of slightly more importance than in the usual type of vessel. Values calculated from the formula of Fox (1909) range from 0.024 at 25° C. to 0.033 at 7.5° C. This term therefore contributes only about 2 per cent to the vessel constant and an error of as much as 10 per cent in the values of α would mean an error of 0.2 per cent in the vessel constant. It is evident then, especially since we are interested in the relative values of oxygen consumption, that errors from this source are negligible.

The manometer readings were made to the nearest 0.5 mm. To determine the pressure changes to which the oxygen consumption data of the following tables correspond it is simply necessary to multiply by the mgm. N of the egg suspension in the vessel and divide by the vessel constant. For example, in Table I, X_{O_2}/mgm. N for the first 20° vessel is 6.7 cu. mm. O_2 in one hour. Multiplying by the mgm. N and dividing by the vessel constant gives 11.5 mm. as the pressure change. Since the reading is made to 0.5 mm. the error is less than 2.5 per cent. This, however, is one of the smallest values presented in the table. If we take the figure 128.8 cu. mm. O_2 from Table II for 5 hours respiration at 20°, this corresponds to a pressure change of 357.5 mm. and the reading error is less than 0.1 per cent.

The measurements were practically all made in duplicate. For the same batch of eggs two vessels were run simultaneously at one temperature and two at a different temperature. Different batches of eggs may vary somewhat in their absolute rates of respiration as well as in their rates of development. To avoid this source of error it is preferable to compare only the results on the same batch of eggs.

Strongylocentrotus

In Table I the results of three experiments with eggs of *Strongylocentrotus* in the early cleavage stages are given. The first column of the table gives the temperature at which the measurements are made;

the second column shows the nitrogen content of the eggs used in each vessel; the third, the age of the eggs as the time from fertilization at 20° C. of the first reading of the manometer; the fourth and fifth give the length of time (from the first reading) during which the oxygen consumption is measured and the corresponding oxygen consumption in cu. mm. O_2 per milligram of egg nitrogen; the sixth and seventh columns again give the respiration time (also from the first reading but continued over a longer time) and the corresponding oxygen consumption.

In the first experiment the Q_5 is 1.36. This was determined from the time between the first and second cleavage for eggs of the same fertilized lot that were placed in the 20° and 15° baths at the same time as the manometers and were also at about the same concentration as the eggs in the manometers. The one-hour interval at 20° therefore corresponds to 1.36 hours at 15° and the five-hour interval at 20° to 6.8 hours at 15°; that is, in those time intervals the same stage of development would be attained at the two temperatures. Comparing then the oxygen consumption for the corresponding time intervals at the two temperatures we see that for both the shorter and longer runs the 20° figures are greater. The differences are, however, within the limits of error. It is evident, too, that for 5 and 6.8 hours the differences are much smaller than for 1 and 1.36 hours, which is to be expected if the Q_5 obtained for the rate of development is reliable, since the errors in the oxygen consumption measurements decrease in the longer run.

TABLE I

Strongylocentrotus; oxygen consumption at different temperatures; early cleavage stages.

Temp.	mgm. N	Time of first reading	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$
° C.		*minutes*	*hours*		*hours*	
20	0.31	45	1.0	6.7	5.0	40.5
20	0.25	45	1.0	6.8	5.0	41.0
15	0.40	45	1.36	6.2	6.8	39.1
15	0.36	45	1.36	6.6	6.8	38.9
20	1.71	35	1.0	6.4	3.5	24.4
20	1.74	35	1.0	6.3	3.5	24.1
10	1.75	35	2.3	6.5	8.1	23.8
10	1.78	35	2.3	6.1	8.1	23.4
20	2.38	50	1.0	6.4	2.5	17.8
20	2.40	50	1.0	6.3	2.5	17.5
7.5	2.39	50	3.2	6.2	8.0	17.6
7.5	2.38	50	3.2	6.3	8.0	17.7

In the second and third experiments larger amounts of material were used and the differences in total oxygen consumption are much smaller. The Q_{10} of the rate of cleavage in the second experiment is 2.3 and the $Q_{12.5}$ in the third experiment is 3.2, both determined, as before, from the time between the first and second cleavage. It may readily be seen that the differences in total oxygen consumption at the two temperatures are no greater than the differences between the duplicates. The two values for one temperature may even lie between the two for the other temperature, as in the third experiment (20° and 7.5° for 2.5 and 8.0 hours).

It may be concluded then that in the early cleavage stages and between 20° and 7.5°, the total oxygen consumed in reaching the same stage of development is the same at different temperatures. We may next see whether this holds for the later stages. In the preceding paper it was shown that the later stages of development were affected in the same manner by temperature as were the cleavage stages. It is possible, however, that the rate of oxygen consumption might be differently affected by temperature in the early cleavage stages and in the later stages. One experiment is presented in Table II and another in Table III showing that this is not the case at least for the temperatures 20° and 15° C. Similar experiments are presented in connection with the other forms investigated.

TABLE II

Strongylocentrotus; oxygen consumption at different temperatures; gastrula stage.

Temp.	mgm. N	Time of first reading	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$
° C.		*hours*	*hours*		*hours*	
20	0.51	25	1.0	23.6	5.0	128.8
20	0.37	25	1.0	24.0	5.0	129.1
15	0.42	25	1.31	20.9	6.55	119.5
15	0.34	25	1.31	21.6	6.55	120.7

In Table II the differences between the average total oxygen consumption at the two temperatures appear to be fairly large compared with the small differences between the duplicates. These differences are, however, most likely due to the value of Q_5 being too small. Only one determination of Q_5 was made for this batch of eggs and it was taken for the time from fertilization to gastrulation, which is less accurately determined than cleavage time. In Table III the differences in oxygen consumption at 20° and 15° are quite small. The figures in this table are arranged differently from those in the other tables and should be compared horizontally. The time interval is given only for the 20° runs. The time interval at 15° is 1.37 times that at 20°. This figure is the Q_5 as determined from the time from the first to second cleavage and from first cleavage to beginning of gastrulation at the two temperatures.

Table III also illustrates the rise in rate of oxygen consumption that occurs as development proceeds. Thus the rate at 21 to 24 hours is three times that at 0 to 3 hours. Of course, even in the first three hours the rate is rising, as may be seen in Table I. It is because of the increasing rate of oxygen consumption that we do not compare the rates at different temperatures, but rather the total oxygen consumed by the eggs in undergoing the same developmental changes at the different temperatures.

TABLE III

Strongylocentrotus; oxygen consumption at different temperatures; cleavage to gastrulation.

Temp.	20°	20°	15°	15°
mgm. N	0.53	0.72	0.61	0.86
Time of first reading	45 min.	18¾ hrs.	45 min.	18¾ hrs.
Time interval at 20° C.	$\frac{x_{O_2}}{\text{mgm. N}}$	$\frac{x_{O_2}}{\text{mgm. N}}$	$\frac{x_{O_2}}{\text{mgm. N}}$	$\frac{x_{O_2}}{\text{mgm. N}}$
hours				
0–3	25.4		24.7	
3–6	34.1		33.8	
6–9	41.8		42.9	
12–15	53.7		55.0	
18–21	65.0	67.2	68.1	68.8
21–24	72.1	74.8	74.9	77.2

A technical point is also illustrated in Table III. It is possible that over long periods of time conditions change in the vessels so that an abnormal respiratory rate is being measured towards the end of a long run. Most of the experiments reported here are of relatively short duration and would not be expected to show such effects. The experiment of Table III shows, however, that reliable data may be obtained throughout a run as long as 24 hours. In this experiment two vessels, one at 20° and one at 15°, were started at 45 minutes after fertilization and run for 24 hours (32.9 hours at 15°). The main batch of eggs was divided between two flasks, one kept at 20° and the other at 15° C. At 18 hours after fertilization (24.7 hours at 15°) embryos from these flasks were washed by gentle centrifugation, and two more respiration vessels, one at 20° and one at 15°, were started. It may be seen from

TABLE IV

Dendraster; oxygen consumption at different temperatures; early cleavage stages.

Temp.	mgm. N	Time of first reading	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$
° C.		*minutes*	*hours*		*hours*	
22	1.28	50	1.0	9.7	3.0	33.5
22	0.82	50	1.0	10.7	3.0	34.6
12	0.92	50	2.8	11.0	8.4	35.9
12	0.82	50	2.8	11.7	8.4	36.6
25	0.69	70	1.0	18.3	3.0	59.1
25	0.53	70	1.0	19.5	3.0	65.8
15	0.62	70	2.2	14.8	6.6	54.9
15	0.78	70	2.2	14.1	6.6	53.0

the table that there are no significant differences in the oxygen consumption for the 18 to 21 and 21 to 24-hour intervals between the two vessels that had been run continuously up to that time and the two that had been freshly started.

Dendraster

It is more difficult with eggs of this form to obtain large quantities in which one hundred per cent are fertilized. In two experiments, how-

TABLE V

Dendraster; oxygen consumption at different temperatures; blastula and gastrula stages.

Temp.	mgm. N	Time of first reading	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$
° C.		*hours*	*hours*		*hours*	
20	0.89	13.0	1.0	34.4	3.0	109.1
20	0.94	13.0	1.0	34.2	3.0	108.1
15	0.76	13.0	1.65	33.5	4.95	105.2
15	0.78	13.0	1.65	34.3	4.95	107.5
15	0.87	12.0	1.0	16.4	3.0	51.0
15	0.98	12.0	1.0	15.9	3.0	49.5
10	0.95	12.0	1.95	16.8	5.85	49.6
10	0.75	12.0	1.95	17.4	5.85	52.0
22	0.21	8.5	1.0	24.4	3.0	75.8
22	0.19	8.5	1.0	25.5	3.0	77.1
12	0.21	8.5	2.75	23.2	8.25	77.6
12	0.20	8.5	2.75	23.7	8.25	78.7
22	0.66	10.0	1.0	27.3	3.0	86.4
22	0.36	10.0	1.0	28.1	3.0	87.0
12	0.55	10.0	2.8	29.4	8.4	92.7
12	0.36	10.0	2.8	31.6	8.4	93.8

ever, fairly large quantities of eggs were obtained in which the fertilization was 95 to 97 per cent. The oxygen consumption data are presented in Table IV, one experiment being at 22° and 12°, the other at 25° and 15°. The Q_{10}'s are 2.8 and 2.2 respectively, determined from the time between first and second cleavage. In the first experiment the oxygen consumption is evidently the same at the two temperatures. In the second experiment the values are higher at 25° than at 15°. The differences appear to be significant, but more data would be needed at these temperatures to establish this point. It should be noted that 25° is just about the upper limit at which normal development is obtained.

In Table V the results of four experiments on blastulæ and gastrulæ are presented, one at 20° and 15°, one at 15° and 10° and two at 22° and 12°. Since only top swimmers are taken, it is not necessary to have 100 per cent fertilization at the start. The time of the first reading (column 3 of the table) represents the age of the embryos at 20° and the corresponding stage of development may be obtained from the data in the preceding paper. The temperature coefficients of the rate of development were determined in these cases from the time between fertilization and the beginning of gastrulation, and the respiration times are, as usual, taken from the values of the temperature coefficients. The oxygen consumption data (X_{O_2}/mgm. N) again show the amount consumed during the same developmental period at the two temperatures of each experiment. As was the case with *Strongylocentrotus,* we find here too no significant differences in the total oxygen consumed at the different temperatures.

An experiment at 20° and 15° covering the entire period from fertilization to gastrulation is presented in Table VI. In this experiment relatively few eggs were used in each vessel and the number was determined at the end of the run by counting them all. There were very few immature eggs in this lot, fertilization being about 98 per cent. In one of the 15° vessels the sea water slopped over into the alkali well. But

TABLE VI

Dendraster; oxygen consumption at different temperatures; from one-cell stage to end of gastrulation.

Temp.	No. of eggs	Time of first reading	Respiration time	$\frac{1000\ X_{O_2}}{\text{No. of eggs}}$	Respiration time	$\frac{1000\ X_{O_2}}{\text{No. of eggs}}$
° C.		*minutes*	*hours*		*hours*	
20	1653	40	6.0	2.2	15.0	7.9
20	2791	40	6.0	2.0	15.0	7.5
15	2581	40	9.6	2.0	24.0	7.4

TABLE VII

Urechis; oxygen consumption at different temperatures; early cleavage stages.

Temp.	mgm. N	Time of first reading	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$
° C.		*minutes*	*hours*		*hours*	
22	1.19	60	1.0	4.4	4.0	18.6
22	1.40	60	1.0	4.9	4.0	21.2
12	1.55	60	2.7	5.9	10.8	23.0
12	2.26	60	2.7	5.2	10.8	21.1
20	1.03	60	1.0	4.0	4.0	17.3
20	1.36	60	1.0	3.9	4.0	17.3
15	1.66	60	1.62	5.0	6.5	18.2

even without the duplicate run at 15° it may readily be seen that the total oxygen consumed at the two temperatures is the same.

TABLE VIII

Ciona; oxygen consumption at different temperatures; cleavage and later stages.

Temp.	mgm. N	Time of first reading	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$	Respiration time	$\frac{X_{O_2}}{\text{mgm. N}}$
° C.		*minutes*	*hours*		*hours*	
20	0.22	60	3.0	28.2	9.0	119.5
20	0.25	60	3.0	29.3	9.0	120.1
15	0.25	60	5.0	28.9	15.0	116.3
15	0.33	60	5.0	27.3	15.0	113.0

Urechis

Two experiments, one at 22° and 12° and one at 20° and 15°, were performed on eggs of *Urechis.* In both 100 per cent fertilization was obtained, as is usual with eggs of this animal. The results are presented in Table VII. The Q_{10} for the rate of cleavage in the 22°–12° experiment is 2.7 and the Q_5 in the 20°–15° experiment is 1.62. In both experiments the total oxygen consumed at the lower temperature is somewhat higher than at the higher temperature. However, considering the variation between the duplicate vessels, and also the error associated with the temperature coefficient determinations for development (see preceding paper), we cannot regard these differences as significant.

Ciona

Two experiments at 20° and 15° were run with eggs of *Ciona.* In the first (Table VIII) a relatively large number of eggs were employed and the amount determined as usual from the Kjeldahl nitrogen. In the second (Table IX), fewer eggs were used and the amount determined by counting.

TABLE IX

Ciona; oxygen consumption at different temperatures; cleavage and unhatched tadpole stages.

Temp.	No. of eggs	Time of first reading	Respiration time	$\frac{1000 X_{O_2}}{\text{No. of eggs}}$	Respiration time	$\frac{1000 X_{O_2}}{\text{No. of eggs}}$
° C.		*minutes*	*hours*		*hours*	
20	1417	60	3.0	1.9	12.0	13.1
20	1273	60	3.0	2.1	12.0	13.3
15	1052	60	5.0	2.1	20.0	12.9
15	1042	60	5.0	2.0	20.0	12.8

There are some technical difficulties in obtaining large quantities of *Ciona* eggs for respiration measurements. Generally several animals must be used and the eggs must be pooled in order to have comparable lots in each vessel. In removing *Ciona* eggs it is very difficult to be certain that no sperm from the same animal is obtained at the same time. This introduces no serious difficulty when eggs from one individual are used since *Ciona* is a self-sterile hermaphrodite. However, to control the time of fertilization when several animals are used it is necessary to mix all the eggs simultaneously and inseminate at the same time. The material of the first experiment was prepared in this manner from four individuals; that for the second experiment came from one individual.

In both experiments fertilization was 100 per cent, which is the rule for *Ciona* eggs removed from the oviduct. The Q_5's as determined from the time from first to second cleavage were 1.67 in both cases. The duplicate runs in these experiments checked very nicely, the biggest difference being 3 per cent. Since the error associated with the temperature coefficients is also of this order of magnitude, it may readily be seen that the differences in total oxygen consumption for the longer runs at the different temperatures are well within the limits of error. For the shorter readings in both experiments the duplicates at the two temperatures overlapped. We may conclude then that in *Ciona,* too, the total oxygen consumed in reaching the same stage of development is the same at different temperatures.

Discussion

In these experiments it is seen that, within the range employed, the effect of temperature on the rate of development parallels its effect on the rate of oxygen consumption. We know, however, that at temperatures at which development is inhibited respiration goes on at a measurable rate. We might therefore expect striking differences as such temperatures are approached. But certain complications enter when we consider inhibiting temperatures. As the temperature is lowered we first reach a point at which cytoplasmic division is interfered with whereas nuclear division proceeds. This point is reached rather abruptly and not after an infinitesimally slow rate of development is attained, as is evident from the fact that in some eggs of a given lot cytoplasmic division is inhibited whereas in others it goes on at an easily measurable rate. For example, in a batch of sand-dollar eggs raised at 7.5° C., about 80 per cent failed to divide while the rest underwent the first cleavage at about 270 minutes after fertilization. The nuclear activity that proceeds when cytoplasmic division has been inhibited must be regarded as a sort of developmental activity. Should we, however, expect the rate of oxygen consumption to drop to zero at temperatures at which all developmental activity has ceased? This is not necessarily the case if there is any significance to the concept of maintenance. If the inhibition by low temperature is reversible then the blocked eggs may be expected to show a basal metabolism characteristic of that temperature. The magnitude need not be the same as that of the unfertilized egg at the same temperature, since, as was pointed out above, the maintenance metabolism of different kinds of cells probably differs. The recent investigations of Whitaker (1931, 1933) bear on this point. Whitaker found that unfertilized eggs of *Chætopterus* and *Cumingia* respire at a higher rate than do the fertilized eggs. If then it were assumed that at any temperature the maintenance component of the developing egg is equivalent to the metabolism of the unfertilized egg, negative values would be obtained for the requirements of the developmental processes. A similar situation arises in one of the interesting series of experiments of Runnström (1935). He finds that the respiration of unfertilized sea-urchin eggs is much more strongly increased on addition of pyocyanine than that of the developing eggs. The eggs may be fertilized in the pyocyanine solution, but the increase in the rate of respiration is very small compared with that in normal sea water. Addition of HCN depresses the rate of the fertilized eggs in pyocyanine but, strangely enough, increases that of the unfertilized eggs, so that at certain concentrations the latter show a higher respiratory rate.

The identity of the temperature coefficients for the rate of development and the rate of oxygen consumption may be interpreted to mean that the maintenance, growth and differentiation requirements are similarly affected by temperature. It might also mean, although it seems more unlikely, that the proportionate requirements for maintenance and growth change but their sum remains the same fraction of the total at the different temperatures. If maintenance is assumed to be the same sort of process, although different in magnitude, in developing eggs and in unfertilized eggs, then, according to the former interpretation, we should expect the respiration of the unfertilized egg to have the same temperature coefficient as that of the fertilized egg. Measurements have been made of the temperature coefficient of the respiration of unfertilized eggs of the sea-urchin by Rubenstein and Gerard (1934). They find it to be different from that of fertilized eggs. The values for the coefficients of the unfertilized egg are higher than for the fertilized eggs, and the big rise in respiratory rate that occurs upon fertilization at ordinary temperatures disappears at higher temperatures. It would appear then that maintenance in unfertilized and in developing eggs is not the same kind of process. There are, of course, a number of assumptions in this argument. The assumption that the same kinds of processes should show identical temperature coefficients is probably the safest of these. But it is also assumed that the respiration of an unfertilized egg is a measure of its maintenance requirement. This is the way in which basal metabolism studies on adults are regarded. But it is quite conceivable that very little, if any, energy is required for maintenance, and that most of that supplied by the oxidations is quite unnecessary. The other assumption is made because, in the absence of any real information as to the nature of maintenance, it appears to be the simplest. For example, the oxygen consumption might be regarded as representing some sort of destructive process in the unfertilized egg (which, in a certain sense, it is, since the metabolites are being destroyed), but calling it destruction is merely a change in terminology. The real point is whether or not the same kind of process occurs in developing eggs.

One conclusion that has been drawn from the dissimilarity of the temperature coefficients of development and respiration (shown chiefly by Loeb and Wasteneys) is that Child's theory of metabolic gradients cannot hold (Crozier, 1926). But regardless of the particular merits of that theory, it is evident that the experimental facts are inadequate to justify that objection. The recent experiments of Brachet (1934) appear, on the other hand, to favor the view that the most "active" regions have the highest metabolic rate. He found the respiratory rate of the dorsal lip of the blastopore in the frog to be higher than that of the rest of the egg, and Child (1929) regards this region as a metabolic center. However, to conclude that the inductive properties of the dorsal lip of the blastopore are due to the high metabolism is certainly premature. As Brachet himself points out, injury to an egg or tissue raises the rate of oxygen consumption, but it seems quite certain that simple pricking of the egg would not give induction. We may await with interest experiments on amphibian gastrulæ in which the effect of local application of various reversible dyestuffs affecting the rate of respiration is tested.

SUMMARY

The effect of temperature on the oxygen consumption of the eggs of four marine invertebrates, *Strongylocentrotus purpuratus, Dendraster excentricus, Urechis caupo* and *Ciona intestinalis,* was investigated.

With *Strongylocentrotus* eggs the measurements were made in the early cleavage and gastrula stages as well as continuously up to the gastrula stage and at the temperatures 20°, 15°, 10°, and 7.5° C.

With *Dendraster* eggs the measurements were made in the early cleavage, blastula and gastrula stages as well as continuously throughout those stages and at the temperatures 25°, 22°, 20°, 15° and 12° C.

With *Urechis* eggs the measurements were made in the early cleavage stages and at the temperatures 22°, 20°, 15,° and 12° C.

With *Ciona* eggs the measurements were made in the early cleavage stages and up to hatching and at the temperatures 20° and 15° C.

In all cases, with the exception of one run with *Dendraster,* the total oxygen consumed during the same developmental period at the different temperatures is the same within the limits of error of the measurements. The exceptional *Dendraster* experiment showed a somewhat higher total respiration at 25° as compared with that at 15° C. Omitting this case, which needs to be further investigated, it may be concluded that at least in the range of temperatures investigated no optimum exists at which development is accomplished with a minimum oxygen consumption.

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LITERATURE CITED

Barthélemy, H., and R. Bonnet, 1926. L'énergie de croissance. IX. Influence de la température sur l'utilisation de l'énergie au cours du développement de l'oeuf de Grenouille rousse (Rana fusca). *Bull. Soc. Chim. biol.,* **8**: 1071.

Brachet, J., 1934. Étude de l'oeuf de Grenouille (Rana fusca) au cours du développement. 3. Metabolisme respiratoire et "centre organisateur" de la gastrula. *Arch. de Biol.,* **46**: 25.

Brown, H. W., 1928. A quantitative study of the influence of oxygen and temperature on the embryonic development of the eggs of the pig ascarid (Ascaris suum Goeze). *Jour. Parasitol.,* **14**: 141.

Child, C. M., 1929. The physiological gradients. *Protoplasma,* **5**: 447.

Child, C. M., 1929. Lateral grafts and incisions as organizers in the hydroid, Corymorpha. *Physiol. Zoöl.,* **2**: 342.

Crescitelli, F., 1935. The respiratory metabolism of Galleria melonella (Bee Moth) during pupal development at different constant temperatures. *Jour. Cell. Comp. Physiol.,* **6**: 351.

Crozier, W. J., 1924. On biological oxidations as function of temperature. *Jour. Gen. Physiol.,* **7**: 189.

Crozier, W. J., 1926. The distribution of temperature characteristics for biological processes; critical increments for heart rates. *Jour. Gen. Physiol.,* **9**: 531.

Dareste, C., 1882. Recherches sur la production des monstres, dans l'oeuf de la poule, par l'effet de l'incubation tardive. *Compt. Rend. Acad. Sci.,* **95**: 254.

Dobzhansky, Th., and D. F. Poulson, 1935. Oxygen consumption of Drosophila pupæ. II. Drosophila pseudoöbscura. *Zeitschr. vergl. Physiol.,* **22**: 473.

Ephrussi, B., 1933. Contribution à l'analyse des premiers stades du développement de l'oeuf. Action de la température. *Arch. de Biol.,* **44**: 1.

Fauré-Fremiet, E., 1924. L'oeuf de Sabellaria alveolata L. *Arch. d'Anat. Micr.,* **20**: 211.

Fox, C. J. J., 1909. On the coefficients of absorption of nitrogen and oxygen in distilled water and sea water, and of atmospheric carbonic acid in sea water. *Trans. Faraday Soc.,* **5**: 68.

Grodzinski, Z., 1933. Über die Entwicklung von unterkühlten Hühnereiern. *Arch. Entw.-mech.,* **129**: 502.

Krogh, A., 1914. On the rate of development and CO_2 production of chrysalides of Tenebrio molitor at different temperatures. *Zeitschr. allg. Physiol.,* **16**: 178.

Lillie, F. R., 1906. Observations and experiments concerning the elementary phenomena of embryonic development in Chætopterus. *Jour. Exper. Zoöl.,* **3**: 153.

Loeb, J., and H. Wasteneys, 1911. Sind die Oxydationsvorgänge die unabhängige Variable in den Lebenserscheinungen? *Biochem. Zeitschr.,* **36**: 345.

McCoy, O. R., 1930. Cited in Needham's Chemical Embryology.

Needham, J., 1933. On the dissociability of the fundamental processes in ontogenesis. *Biol. Rev.,* **8**: 180.

Rubenstein, B. B., and R. W. Gerard, 1934. Fertilization and the temperature coefficients of oxygen consumption in eggs of Arbacia punctulata. *Jour. Gen. Physiol.,* **17**: 677.

Runnström, J., 1935. On the influence of pyocyanine on the respiration of the sea urchin egg. *Biol. Bull.,* **68**: 327.

Tyler, A., 1933. On the energetics of differentiation. A comparison of the oxygen consumption of "half" and whole embryos of the sea-urchin. *Publ. Staz. Zool. Napoli,* **13**: 155.

Tyler, A., 1935. On the energetics of differentiation. II. A comparison of the rates of development of giant and of normal sea-urchin embryos. *Biol. Bull.,* **68**: 451.

Tyler, A., 1936. On the energetics of differentiation. III. Comparison of the temperature coefficients for cleavage and later stages in the development of the eggs of some marine animals. *Biol. Bull.,* **71**: 59.

Warburg, O., 1926. Stoffwechsel der Tumoren. Berlin.

Whitaker, D. M., 1931. On the rate of oxygen consumption by fertilized and unfertilized eggs. II. Cumingia tellinoides. *Jour. Gen. Physiol.,* **15**: 183.

Whitaker, D. M., 1933. On the rate of oxygen consumption by fertilized and unfertilized eggs. IV. Chætopterus, and Arbacia punctulata. *Jour. Gen. Physiol.,* **16**: 475.

Whitaker, D. M., 1933. On the rate of oxygen consumption by fertilized and unfertilized eggs. V. Comparisons and interpretation. *Jour. Gen. Physiol.,* **16**: 497.

PARTHENOGENETIC MEROGONY OR CLEAVAGE WITHOUT NUCLEI IN ARBACIA PUNCTULATA

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Parts of unfertilized *Arbacia* eggs from which the nucleus has been removed, may be activated by parthenogenetic agents, and they will throw off fertilization membranes, and cleave quite like the fertilized nucleate eggs. This combination of parthenogenesis, in which the male nucleus is lacking, and merogony (development of a fertilized enucleate egg fragment), in which the female nucleus is lacking, may be termed "parthenogenetic merogony." It is of particular interest since the resulting non-nucleate organism lacks chromosomes, genes and therefore the hereditary qualities usually associated with the nucleus.

MATERIAL—THE PARTS OR FRACTIONS OF CENTRIFUGED EGGS

The material has been obtained by breaking apart the mature unfertilized eggs of *Arbacia punctulata* by centrifugal force and using the non-nucleate parts. The method, as previously described (E. N. Harvey, 1931; E. B. Harvey, 1932) consists in centrifuging the eggs in a sucrose solution of the same tonicity and approximately the same density as the eggs. The eggs remain suspended in this solution during centrifugation and are free to elongate and break apart. The stratification of the centrifuged egg is shown in Plate I, Fig. 1, and eggs in the process of stratification and breaking, as they are observed with the centrifuge microscope, are shown in Photograph 1.

The eggs become dumb-bell shape and then usually break into two slightly unequal parts, a larger *white half* containing oil, clear layer, mitochondria and some yolk, and a smaller *red half* containing yolk and pigment (Figs. 2, 6 and Photographs 2, 4).[1] The nucleus is always in the white half under the oil—this is *invariable* in *Arbacia punctulata*. Both the halves will elongate with further centrifuging (Figs. 3, 7 and Photographs 3, 5) and break into quarters; the white half breaks into a larger *clear quarter* containing oil, clear layer and nucleus and practically

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[1] The sizes of the individual granules are: oil (spherical droplets) 0.6–1.0 μ; mitochondria (fine granules) 0.6–1.0 μ; yolk (irregular or polyhedral granules) 0.7–1.1μ; pigment (spherical granules) 1.1–1.6 μ. The relative amount of formed bodies as ascertained by E. N. Harvey (1932) is: oil 1 per cent; mitochondria 4.8 per cent; yolk 27.2 per cent; pigment 5.5 per cent.

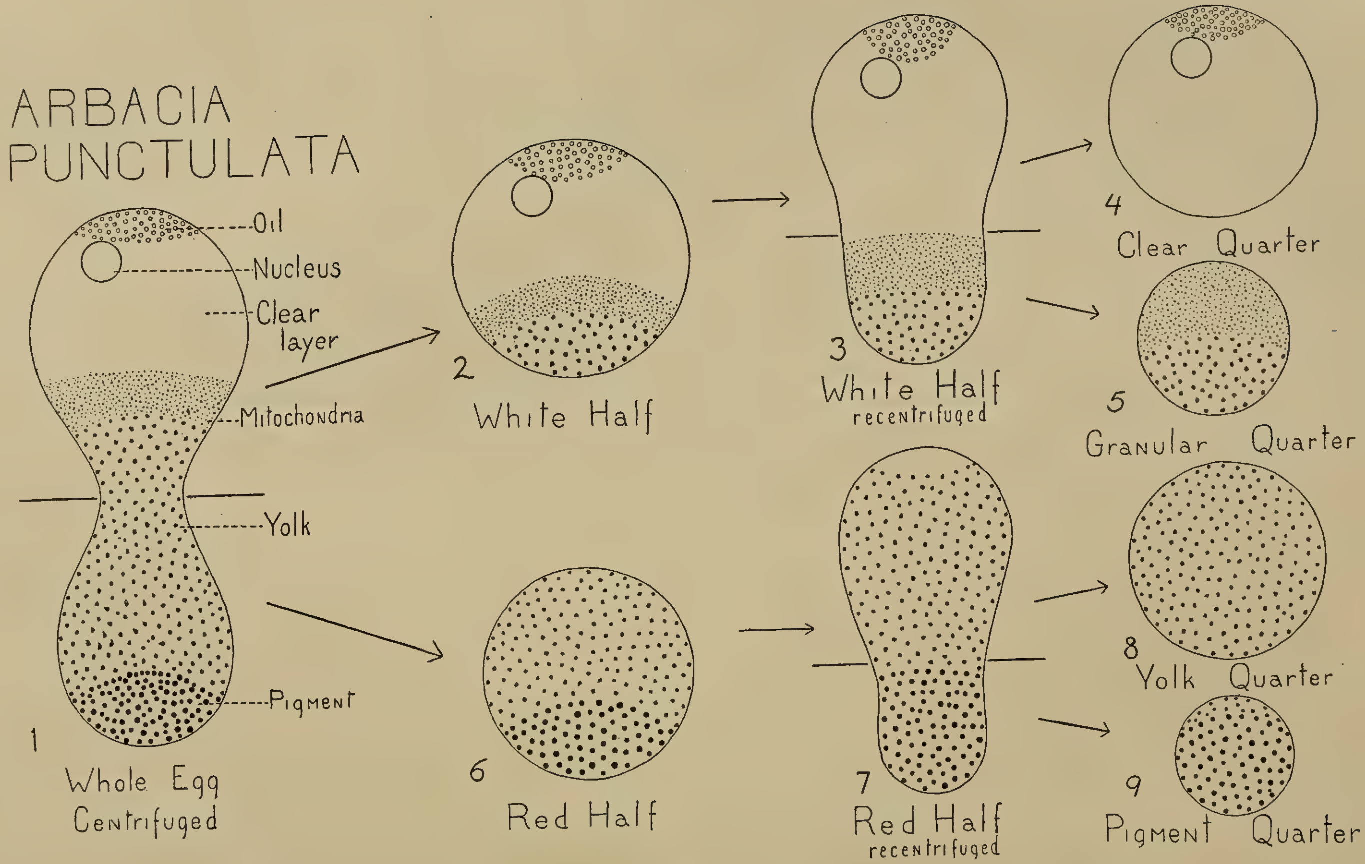
ARBACIA
PUNCTULATA
Oil
Nucleus
Clear layer
Mitochondria
Yolk
Pigment
1
Whole Egg Centrifuged
2
White Half
3
White Half recentrifuged
4
Clear Quarter
5
Granular Quarter
6
Red Half
7
Red Half recentrifuged
8
Yolk Quarter
9
Pigment Quarter

PLATE I

The unfertilized egg of *Arbacia punctulata,* stratified by centrifugal force (about 3 minutes at 10,000 × g.), and the halves and quarters into which it breaks. The drawings are from camera lucida sketches and photographs, made as accurately as possible to scale. Magnified 500 ×.

The clear area in Fig. 7 at the centripetal pole is due to further packing of the granules with longer centrifuging.

no visible granules, and a smaller *granular quarter* containing all the mitochondria and some yolk granules (Figs. 4 and 5 and Photograph 3). The nucleus is invariably in the clear quarter. The red half egg breaks into a larger *yolk quarter* containing only yolk granules, and a very small *pigment quarter* containing all the pigment and a few yolk granules (Figs. 8, 9 and Photographs 1, extreme right, and 5). The halves and quarters are of a very definite and characteristic size and remarkably uniform in any particular batch of eggs (i.e. eggs from one female). The sizes of the halves and quarters (which I will call " fractions ") of typical batches are given in Table I; the table is based on the measurements of many eggs from many typical batches and may be considered as a standard.[2] The four parts or fractions which lack a nucleus are marked with asterisks; they are (1) granular quarter, (2) red half, (3) yolk quarter, (4) pigment quarter. There is no possibility of any of these fractions containing any portion of the female nucleus, since in *Arbacia* this is always thrown intact under the oil cap.

TABLE I

Size of the *Arbacia* egg and its fractions obtained by centrifugal force.

Parts	Diameter	Volume	Approximate proportion of whole egg
	μ	μ^3	*per cent*
Whole egg	74	212,000	
White half	62	125,000	60
Clear quarter	56	92,000	40
* Granular quarter	40	33,500	20
* Red half	56	92,000	40
* Yolk quarter	52	73,600	33
* Pigment quarter	32	17,160	7
(Nucleus	11.5	796	0.4)

* Non-nucleate.

Though the great majority of batches of eggs conform quite closely to the sizes given in Table I, there are some consistent variations. A few batches occur each year in which there is a great disparity in the size of the halves, the red halves being very small (Photograph 7); this occurs uniformly throughout the batch irrespective of the speed of centrifuging. Then there occur a few batches which break into almost equal parts, through the mitochondrial layer (Photograph 8).[3] Then there occur in the course of the summer's work one or two batches in which the eggs of one batch break at two levels, approximately half of the eggs break into a very small red half and large white half, and the others into a larger red half and a smaller white half (Photograph 9); this gives white halves (and of course the corresponding red halves) of two distinct sizes (Photograph 10). These variations from the standard type of breaking must be due to a difference in the physical structure of the egg, probably viscosity. Such would seem to be the case since the stratification varies with the position of the break; the mitochondrial layer is much better formed when the two halves are nearly equal (*cf.* Photographs 7 and 8). Apart, however, from these infrequent and consistent variations, the material is quite uniform, and there can be no question as to the structure of the various fractions, especially as to whether they are nucleate or not.

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[2] These figures for the relative size of the halves agree closely with those of Shapiro (1935) for slightly smaller eggs.

[3] The measurements of Lucké (1932) to determine the distribution of osmotically inactive material, include two sets (*B* and *D*) of this unusual type.

Method

For the experimental work, the eggs are centrifuged in tubes a little larger than hæmatocrit tubes, of about 0.7 cc. capacity. The eggs in sea water are placed above a layer of 0.95 molal sucrose solution in the proportion of about ⅓ sea water to ⅔ sucrose solution; this is isosmotic and isopycnotic with the eggs. After centrifuging for 3 to 4 minutes at about 10,000 × gravity, there are usually three distinct layers in the tubes, a layer of white halves above, a pinkish layer of elongate but as yet unbroken whole eggs a little below, and a layer of red halves at the bottom of the tube (Photograph 6, right). With further centri-

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Explanation of Photographs 1–6

Centrifuged egg and egg fractions of *Arbacia punctulata*

1. Unfertilized *Arbacia* eggs stratifying and pulling apart as observed in the centrifuge microscope. Photographed while rotating at about 10,000 × gravity. At the bottom of the slide are the red non-nucleate halves. At the right is one of the red halves pulling apart into quarters; at the extreme right, the yolk quarter above and the pigment quarter below.

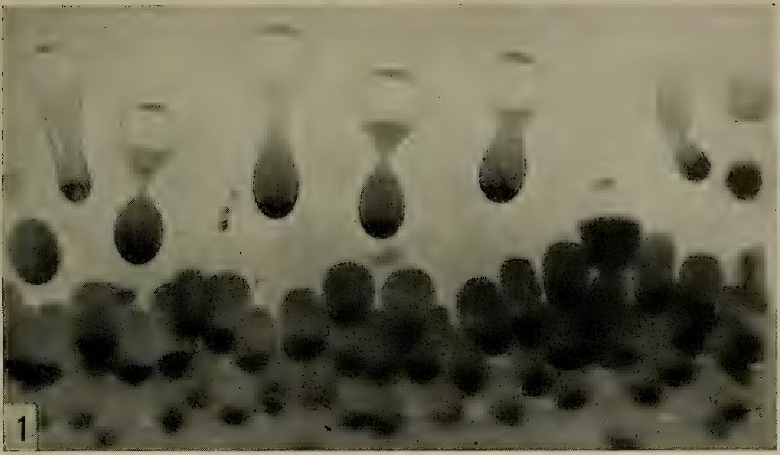
2. White halves showing oil cap (black), clear layer, and granules (mitochondria and yolk). The nucleus invariably lies under the oil cap.

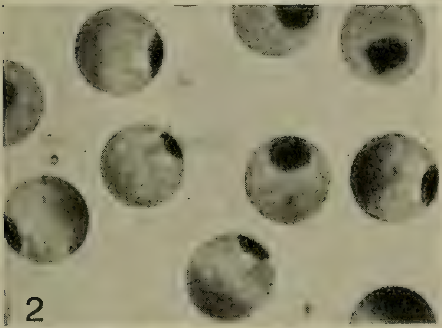
3. White halves (at right and left) elongated by greater centrifugal force, and their two quarters, clear quarter (below) with nucleus under the oil cap, and granular quarter (above).

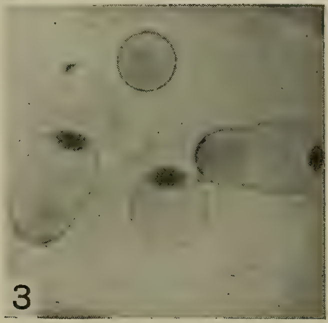
4. Red halves, non-nucleate, containing only yolk and pigment (black).

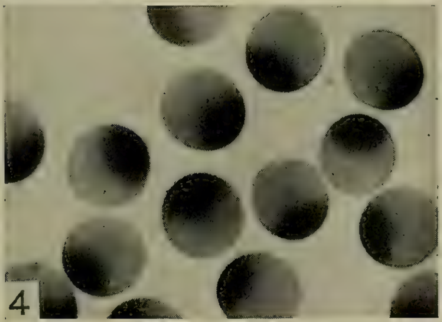
5. Red halves elongated by greater centrifugal force, separating into their two quarters, yolk quarter (larger and lighter) and pigment quarter (smaller and darker).

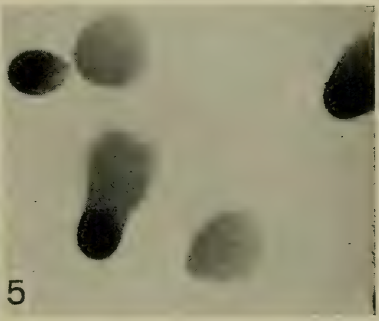
6. Centrifuge tubes (placed upright immediately after removal from the centrifuge), showing masses of eggs and egg fractions in distinct layers. Right tube, three layers: (above) white halves; (center) elongate but as yet unbroken whole eggs; (bottom) red halves. Left tube, five layers: (1, at top) white halves; (2) unbroken whole eggs; (3) yolk quarters; (4) red halves; (5, at bottom) pigment quarters.

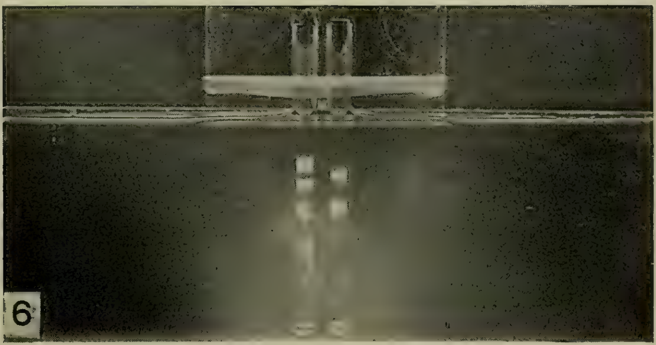




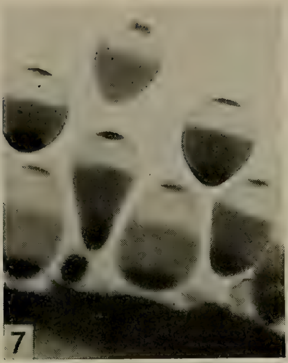








Photographs 1–6



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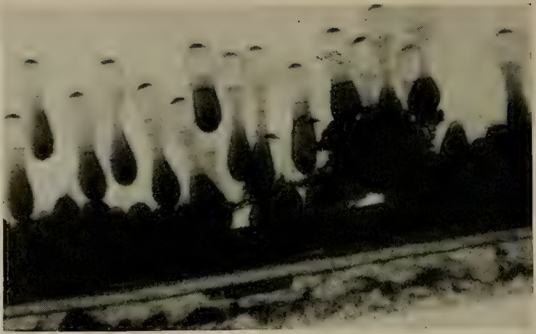

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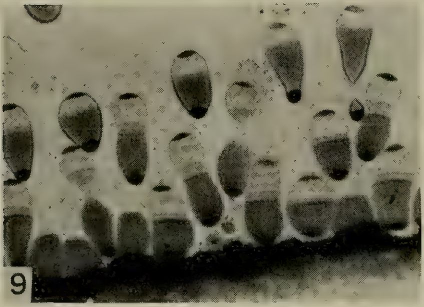

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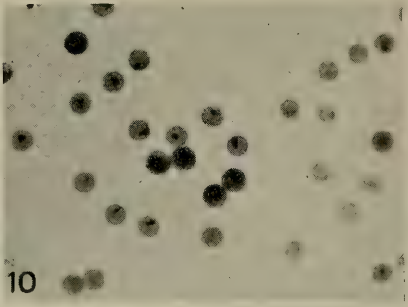

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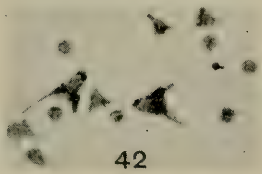

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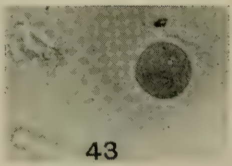

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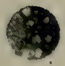

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PHOTOGRAPHS 7–10

PHOTOGRAPHS 7–10 show unusual breaking of unfertilized *Arbacia* eggs. PHOTOGRAPHS 7–9 were taken while rotating on the centrifuge microscope.

7. One broken egg shows unusual breaking into a very large white half (above) and a very small red half (below). Mitochondrial layer not well formed.

8. Unusual breaking into almost equal halves. Mitochondrial layer well formed, and the break comes across this layer.

9. Unusual breaking into two types in the same batch of eggs: (1) small white and large red halves at left and center; (2) large white and small red half at right. Nucleus shows well in some of these eggs.

10. Lower magnification of white halves of two distinct sizes, no intermediates, from such a batch as shown in Photograph 9. The larger ones appear darker because of the mass of granules; these obscure the oil cap, which is plainly visible in the smaller type.

42. Plutei and blastulæ from whole and white half eggs, fertilized, 2 days old. Several white halves have developed into plutei half the normal size. Other white halves are still spherical blastulæ. (Low magnification.)

43. Parthenogenetic merogone from yolk quarter, 4 weeks old. Detailed drawing of same in Fig. 20, Plate II. Highly magnified.

44 and 45. Fertilized merogones. 44. Fertilized yolk quarter with 2 daughter nuclei. Cell division only indicated by notch at left. Three hours after fertilization. 45. Fertilized red half with about 12 nuclei and no cell boundaries. Five hours after fertilization.

fuging and using more of the sucrose solution, the red halves are often broken into quarters and there are five distinct layers in the tubes: (1) white halves, (2) elongate whole eggs, (3) yolk quarters, (4) red halves, (5) pigment quarters at the bottom (Photograph 6, left). One can decant off from the tubes the two upper layers of cells, which contain nuclei, and obtain a pure culture of non-nucleate fractions. Thus is obtained the material for the study of parthenogenetic merogony, and it can be obtained in great abundance. The study has been made almost exclusively on living eggs and all photographs and drawings are from living material. I have not yet worked with the granular quarters of the white halves; these are more difficult to obtain as it requires prolonged centrifuging. The yolk quarters have proved to be excellent material for the observation of cytological details in the living eggs, owing to their lack of pigment. Even the pigment quarters, though only about one-fourteenth the volume of the whole egg, are activated and cleave.

PARTHENOGENETIC AGENTS

For parthenogenetic agents, I have used, for the most part, hypertonic solutions: sea water concentrated by boiling to half its volume, or sea water brought to a similar hypertonicity by the addition of NaCl in the proportion of 30 grams of NaCl to a liter of sea water. The addition of KCl to sea water (39 grams to a liter) is efficacious but renders the surface of the egg sticky. Also leaving the eggs in the sucrose solution for an hour or keeping them for 24 hours at 8° C. will initiate development. The eggs are left in the hypertonic solution for twenty minutes, and then returned to sea water. This procedure causes development in both nucleate and non-nucleate fractions, but the different fractions, and also the whole eggs, from the same batch react quite differently. The same solution that activates the red halves may fail to activate the white halves or the whole eggs, and usually when the white halves develop the red halves do not. The batch, however, runs quite uniformly; if some of the red halves are activated, the majority of them are; it is not sporadic. Stretched eggs are activated more readily than unstretched, probably because the membrane is thinner and materials can pass through more easily. The stretched whole egg is activated more readily than the normal uncentrifuged egg, and elongate fractions are activated more readily than the same fractions which have become spherical on standing. The eggs are treated, therefore, immediately after removal from the centrifuge. It may be that the sucrose solution in which the eggs are centrifuged helps the activation.

DEVELOPMENT OF NON-NUCLEATE FRACTIONS (PARTHENOGENETIC MEROGONES)

The first sign of activation of any of the centrifuged fractions or whole eggs takes place while in the hypertonic solution; it is a " setting " of the egg. The egg retains its elongate shape and the granules do not redistribute. This is characteristic also of centrifuged whole eggs *fertilized* immediately after centrifuging; they retain their dumb-bell shape and the fertilization membrane follows the irregular contour of the egg (E. B. Harvey, 1932). If, however, they are not fertilized immediately after removal from the centrifuge, but are left unfertilized in sea water, they become spherical within an hour and the granules redistribute. Centrifuged whole eggs and nucleate fractions when activated artificially are also " set."

The non-nucleate fractions are likewise " set " (Photograph 11). In the best batches nearly 100 per cent are " set." Then within ten or fifteen minutes, they usually throw off a fertilization membrane and the ectoplasmic layer forms. In some batches, the fertilization membrane does not form, but development proceeds nevertheless. If kept in the hypertonic solution, the eggs remain in the same condition for some hours with no further development.

After treatment with hypertonic sea water for 20 minutes, the non-nucleate eggs are returned to sea water. Frequently the change from the hypertonic solution to the sea water causes a rupture of the fertiliza-

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EXPLANATION OF PLATE II

Detailed camera lucida drawings of non-nucleate egg fractions (parthenogenetic merogones) from living material. The coarse stippling denotes yolk granules, the large solid dots denote pigment. (The times given are times after activation and return to sea water.)

FIG. 10. Formation of clear area (astrosphere) in non-nucleate yolk quarter. One hour after return to sea water.

FIG. 11. Formation of monaster in non-nucleate yolk quarter. Two hours after return to sea water.

FIG. 12. Formation of amphiaster.

FIGS. 13, 14. First cleavage.

FIG. 15. Non-nucleate red half with many asters and no cell boundaries. " Crater blastula." Still within fertilization membrane.

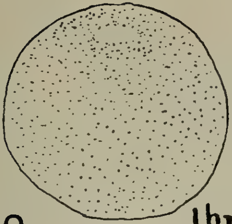
FIG. 16. Cell boundaries have come in around the asters.

FIG. 17. Embryo emerges from the fertilization membrane as individual cells which later disintegrate.

FIG. 18. Three-day non-nucleate blastula from yolk quarter; large cells inside, small cells outside.

FIG. 19. Eight-day non-nucleate embryo from red half, vacuolated. Not divided into cells.

FIG. 20. *A*. Four-week non-nucleate embryo from yolk quarter, not divided into cells, but with vacuoles and large spherical inclusions. *B*. More detailed drawing of the surface, showing possible cilia. Photograph 43 is of this same embryo, still living.



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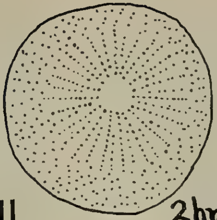

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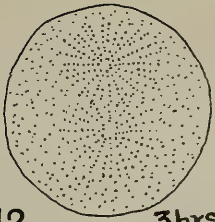

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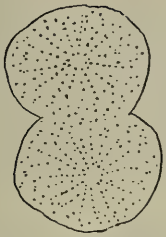
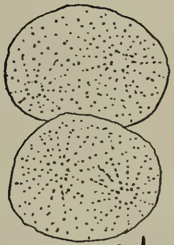
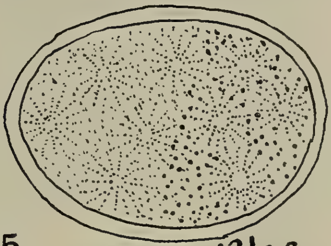


13 4 hrs.



14 6 hrs.



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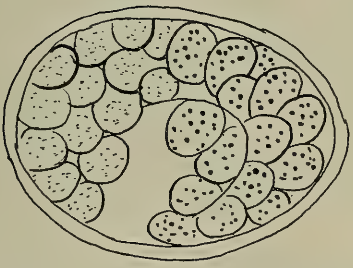
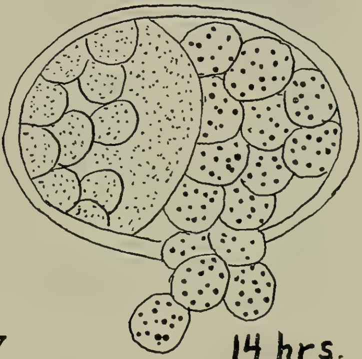
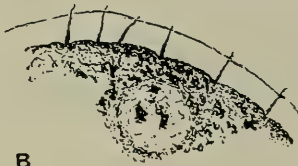


16 13hrs.



17 14 hrs.



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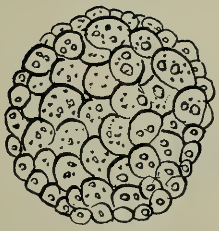
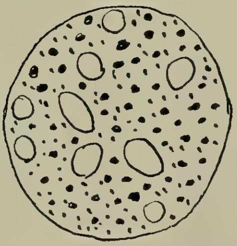
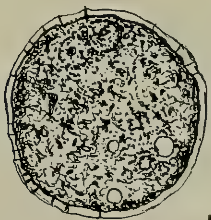


18 3 days

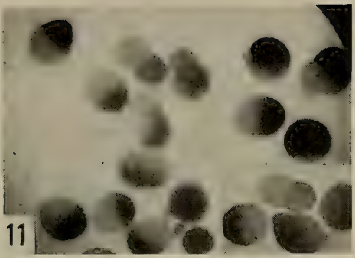


19 8 days



A 20 1 month

Plate II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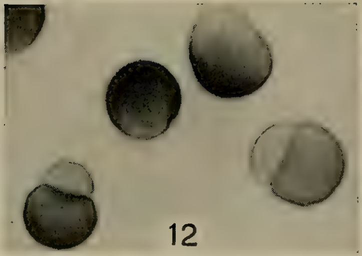
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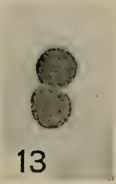
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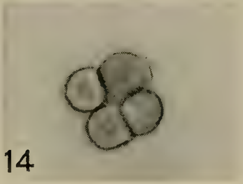
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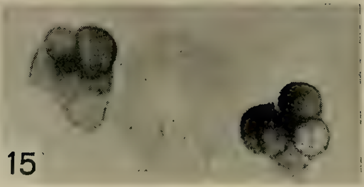
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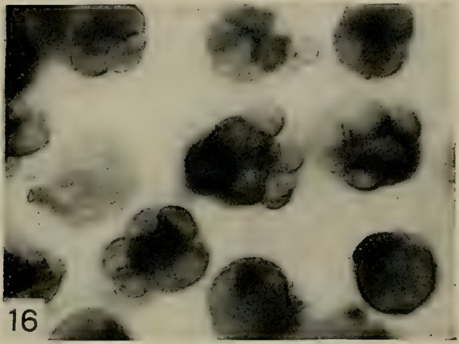
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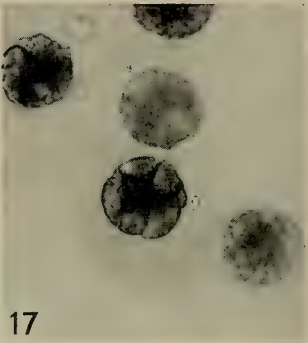
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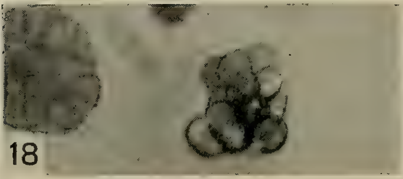
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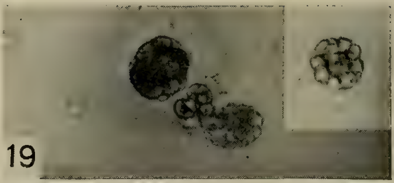
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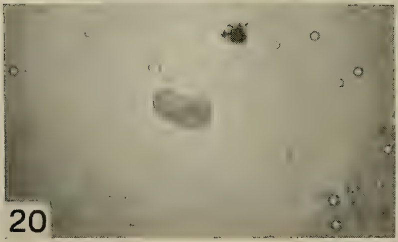
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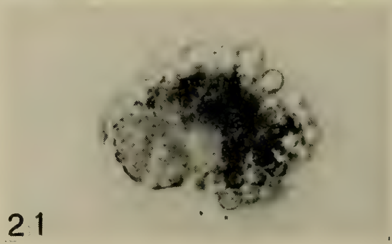
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PHOTOGRAPHS 11–21

All are of living non-nucleate fractions (parthenogenetic merogones)

11. One- and 2-cell stage of red halves; two cells nearly equal in size. Five hours after activation and return to sea water.

tion membrane, owing to the swelling of the egg. After about an hour, a clear area may be observed in the living eggs, especially in the yolk quarters and in the yolk section of the red halves; this is an astrosphere, the beginning of a cytaster (Plate II, Fig. 10). After another hour an aster is formed around this, forming a large and quite typical monaster (Fig. 11). After another hour one often observes two cytasters (Fig. 12), and this may be followed by a cleavage plane between the two asters in quite typical fashion (Figs. 13, 14). A spindle is not visible in the *living* material in these non-nucleate eggs nor indeed in any mitotic division of fertilized eggs. A thorough study of prepared and sectioned material has not yet been made, but a survey of a few slides has failed to reveal anything but asters in the parthenogenetic merogones; these are very granular along the rays.

The activated non-nucleate fractions may divide into two equal or unequal cells (Photographs 11, 12, 13). In the best batches, about eighty per cent cleave. The cleavage plane in the red halves is not constant in position; it often comes in along the boundary between yolk and pigment, but this is not always so. It usually comes in across the narrow axis of an elongate egg, but often not the shortest axis. Very frequently, the egg is pinched in by the free edge of the now hardened fertilization membrane which had broken (usually at the centripetal pole) when the egg was taken from the hypertonic solution to sea water; the cleavage plane usually comes in above this constriction (Photographs 26, 36). This gives a very striking and characteristic 2-cell stage. Such a 2-cell stage is found also in elongate whole eggs treated parthenogenetically (Photograph 31), and also in elongate whole eggs fertilized immediately after centrifuging (Photograph 30). In the latter case, however, the constriction is the original narrowed portion

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12. One- and 2-cell stage of red halves; two cells unequal; fertilization membrane present. Five hours after activation.

13. Two-cell stage of yolk quarter. Six hours after activation.

14. Four-cell stage of yolk quarter. Asters faintly visible, one in each cell. Five hours.

15. Six- and 8-cell red half. Four hours.

16. Eight- to 16-cell red halves. Five hours.

17. Eight- to 16-cell red halves. Cells still confined within fertilization membrane. Eight hours.

18. About 24-cell red half. Sixteen hours.

19. About 64-cell red half. Blastula in center breaking through the fertilization membrane, others still enclosed. Twelve hours.

20. Yolk quarter (not divided into cells), emerging from the fertilization membrane in amœboid fashion. Empty fertilization membrane at right. One and one-third days.

21. Yolk quarter, divided into many cells; about 500 in number. Two days.

of the egg preceding the break into two spheres which would have occurred with further centrifuging; it is not caused by a broken fertilization membrane as in the parthenogenetic eggs. In any of these cases, however, it is more usual for the cleavage plane to come in above (centripetally to) the constriction and not through it, determined in all probability by the stratification. The separation between the two non-nucleate cells is a bona-fide cleavage plane and not merely a pinching in of the surface, as is evident from Photographs 26 and 27—the latter taken two minutes after the former.

A second cleavage plane may divide the two non-nucleate cells into four, again between two asters (Photograph 14; asters are visible in Photographs 36 and 14), or it may divide only one of the first two blastomeres, giving a 3-cell stage. Or the non-nucleate egg may occasionally divide at once into 3 or 4 cells. By subsequent cleavages more cells are formed, and there occur fairly regular 8-, 12- and 16-cell stages, and intermediate numbers. Sometimes these are still enclosed in the fertilization membrane (Photograph 17); sometimes they form a loose cluster of cells not confined within a membrane (Photographs 15, 16, 18, 24, 25, 28, 29, 37, 38). Cell boundaries sometimes disappear and new cell boundaries are formed. I have not found any indication of micromeres at the 16-cell stage of the non-nucleate egg, but these apparently do not form even in the whole eggs fertilized immediately after centrifuging, where the cleavage is quite irregular owing to the shape of the egg. The cleavages of the yolk quarters are more regular than those of the red halves, probably because they lack the heavy red pigment and are more homogeneous and more nearly spherical. The pigment cells of the red halves are usually slower to divide than the yolk cells and are therefore, in general, larger. These same facts are true also of similar fertilized fractions.

Even the pigment quarters, though only about one-fourteenth the volume of the whole egg, will cleave when activated. They often divide

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Explanation of Photographs 22–29

All are of living non-nucleate fractions (parthenogenetic merogones)

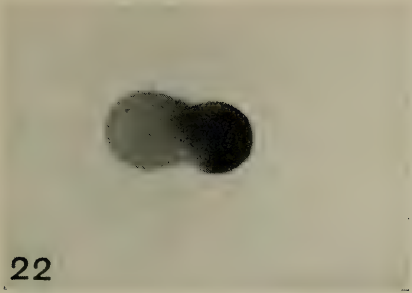
22–25. Series from one and the same red half egg. Times denote hours after activation and return to sea water.

26. Red half 1½ hours after activation. First cleavage plane (at left) beginning through shorter axis. The constriction at right is caused by the pinching in of the broken and now hardened fertilization membrane, and is not a cleavage plane.

27. Same egg 2 minutes later, showing further progress of cleavage plane.

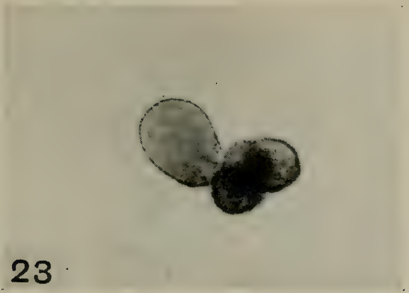
28. At right, 3-cell stage of pigment quarter. At left, about 8-cell stage of red half. Six hours after activation.

29. At right, below, 4-cell stage of pigment quarter. Above, about 6-cell stage of red half. Six hours after activation.



22

1½ hrs.



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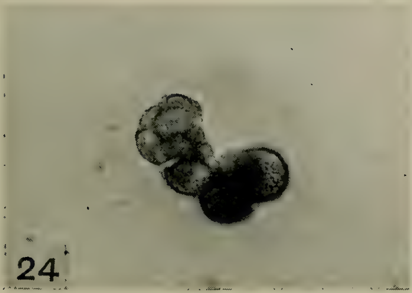
4 hrs.



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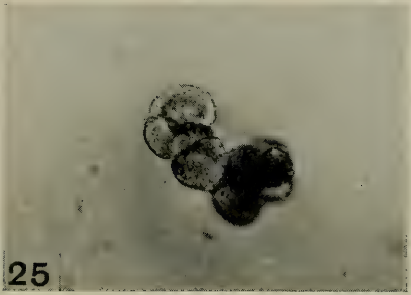
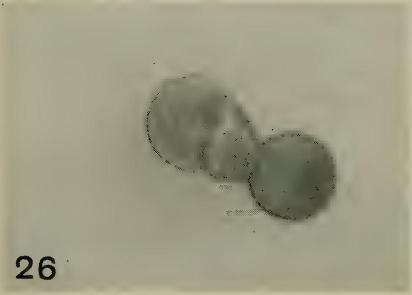
5 hrs.



25

5¼ hrs.



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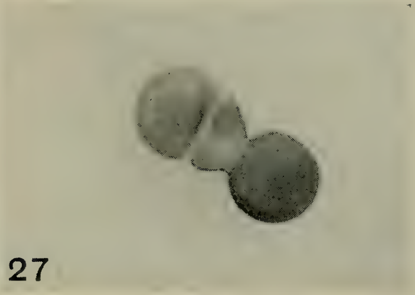

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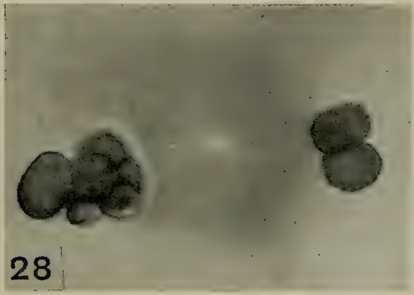

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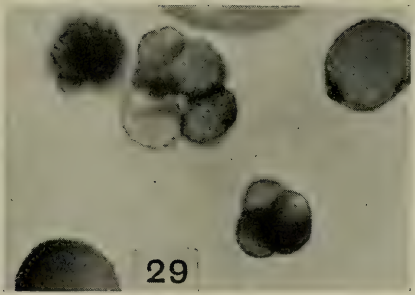

29

Photographs 22–29

into two equal cells, and by subsequent cleavages into as many as 16 cells (Photographs 28, 29, 37). These unfertilized activated quarters have in my experiments cleaved better than the fertilized pigment quarters; I have obtained the latter with 16 nuclei but without cell boundaries.

It should be especially noted that cleavage follows upon cleavage in the non-nucleate egg in a fairly regular sequence. Photographs 22–25 are a series from the same egg at various intervals. The cleavages are not so regular as in normal uncentrifuged fertilized eggs, where they occur with clock-like regularity, but they are almost as regular in occurrence as in fertilized or parthenogenetic centrifuged eggs and in fertilized fractions. Indeed the similarity of the cleavage figures in the non-nucleate egg fractions to those in nucleate ones or in nucleate whole eggs is very striking. The cleavage of a centrifuged egg fertilized immediately after centrifuging is different from that of a normal fertilized egg owing to its elongate shape and to the uneven distribution of granules. The cleavage of an elongate stratified non-nucleate egg fraction is precisely like that of a similar nucleate fraction. Whether

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Explanation of Photographs 30–41

Two vertical columns arranged for comparison of nucleate (30–35) with non-nucleate (36–40) eggs at similar stages of development.

30. Two-cell stage of whole egg fertilized immediately after centrifuging. One hour after fertilization.

31. Two-cell stage of whole egg, unfertilized but artificially activated immediately after centrifuging. Four hours after activation.

32. Below, 2-cell stage of pigment quarter. Above, 8-cell stage of yolk quarter. Three hours after fertilization.

33. Sixteen- to 32-cell stage of whole eggs 3 hours after fertilization. At right, the two components from the first two cells have developed separately without fertilization membrane; with further development these would produce twins.

34. Early blastulæ 4 hours after fertilization. At left, whole egg. At right, white half, unusually large. Still within fertilization membranes.

35. Late blastula from yolk quarter, 1 day after fertilization.

36. Two-cell stage of non-nucleate red half. Note two asters in upper cell. Six hours after activation.

37. Above, at left, 8-cell stage of non-nucleate yolk quarter. Above, center, 2-cell stage of pigment quarter. Below, right, 8-cell stage of red half. All non-nucleate. Five hours after activation.

38. Sixteen- to 32-cell red halves, non-nucleate. Nine hours after activation. At right, the two components from the first two cells have developed separately; with further development these would produce twin blastulæ.

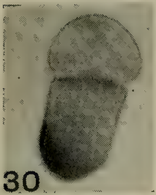
39. Early non-nucleate blastulæ, 12 hours after activation. At left, red half. At right, yolk quarter. Still within fertilization membranes.

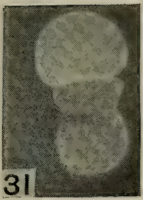
40. Late non-nucleate blastula from yolk quarter, 2 days after activation.

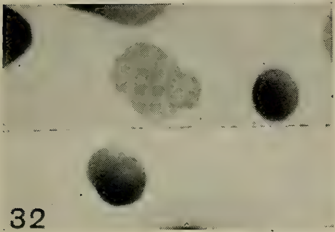
41. Left, pluteus from whole egg, 3 days after fertilization. Right, above, small blastula from non-nucleate yolk quarter, 3 days after activation. To show comparative size and differentiation after 3 days.

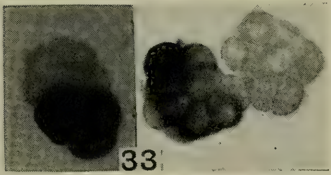
42–45 follow Photograph 10.

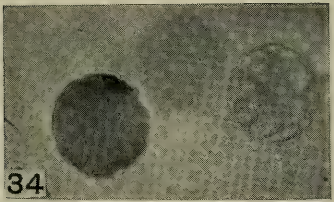
NUCLEATE

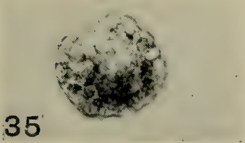

30
Fertilized
1 hr


31
Parthenogenetic
4 hrs

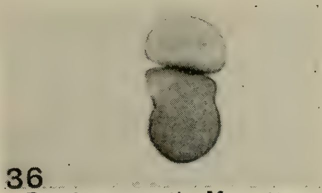

32
3 hrs

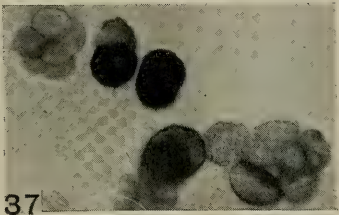

33
3 hrs

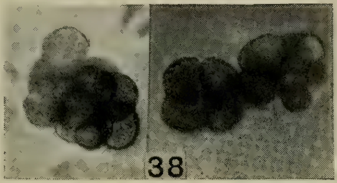

34
4hrs

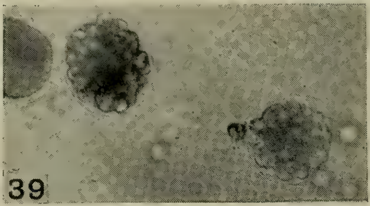

35
1 day

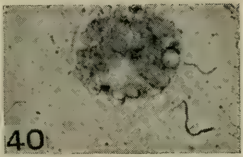
NON-NUCLEATE

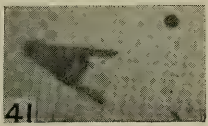

36
Parthenogenetic Merogone
6 hrs


37
5 hrs


38
9 hrs


39
12 hrs


40
2 days


41
3 days

Photographs 30–41

the nucleate egg or fraction has one or two nuclei, that is, whether it is fertilized, or parthenogenetic, or merogonic, makes little difference in the type of cleavage. Compare, for instance, the 2-cell stage of a fertilized whole egg (Photograph 30), a parthenogenetic whole egg (Photograph 31), and a non-nucleate red half (Photograph 36). The first cleavage, as mentioned previously, in an elongate egg usually comes in across the shorter axis in the less dense area. Later cleavages of nucleate and non-nucleate eggs are closely similar, as a comparison of Photographs 32–35 with those of Photographs 37–40 will show. Cleavage stages of nucleate eggs are at the left and the corresponding stages of non-nucleate ones at the right. Even the frequent independent development of the first two blastomeres is similar (Photographs 33 and 38). The main difference is in the rate of cleavage. The non-nucleate eggs cleave much more slowly. Whereas it takes about fifty minutes (at 23° C.) for the first cleavage of a fertilized whole egg, whether normal or centrifuged, and the same time for the fertilized white half, it takes about six hours after activation for the first cleavage of a non-nucleate egg. The times for first cleavage as well as later ones are extremely variable in different sets of the non-nucleate eggs. A nucleate egg, treated parthenogenetically, is intermediate in rate; it takes about four hours after activation for the first cleavage. The merogonic egg fractions, i.e., fertilized enucleate parts of eggs, are also slow to cleave (Photograph 32; see also E. B. Harvey, 1932). In general, eggs with 2 nuclei (♂ and ♀) develop most rapidly, those with one (♂ or ♀) less so and those without any nucleus most slowly; the very slow development of the fertilized clear quarter is an exception (1932). It may be that the nucleus contains an enzyme which accelerates cleavage, but apparently certain granules must be present.

Later cleavages of non-nucleate fractions (parthenogenetic merogones) result in the formation of a blastula. The blastula, if still confined within the fertilization membrane, may be observed breaking through the membrane after about twelve hours (Photographs 19, 39). It frequently happens that cleavage planes do not come in, especially in the dense red halves, and more and more cytasters appear as time goes on, giving the surface somewhat the appearance of the surface of the moon, pitted by many craters (Fig. 15). Exactly the same phenomenon was noted in the development of the fertilized red half-eggs (Fig. 20, 1932), only here nuclear membranes are present. Photographs 44 and 45 are of fertilized enucleate fractions showing a multinucleate condition without cell boundaries. Cleavage planes frequently come in, in these red halves both fertilized and non-nucleate, just before the breaking of the fertilization membrane, thus dividing the egg into many cells

(Fig. 16). The non-nucleate blastula sometimes emerges as isolated cells instead of as a complete organism, and then goes to pieces (Fig. 17). Sometimes the cleavage planes do not come in even at this late stage, and the embryo emerges from the fertilization membrane as a compact mass of protoplasm, somewhat amoeboid in appearance, but perfectly viable (Photograph 20). The oldest cellular non-nucleate blastulæ obtained were from yolk quarters two and three days old (Fig. 18, Photographs 21, 40). These consisted of somewhat differentiated cells, the inside cells larger than the peripheral; the individual cells were filled with granules and small vacuoles or fat droplets. As many as 500 cells have been counted in these blastulæ. Staining *in toto* with aceto-carmine failed to reveal nuclei or chromosome plates which are well brought out in fertilized yolk quarters similarly treated. The embryos usually do not survive more than three or four days owing both to their low vitality and to the accumulation of decomposing eggs in the culture with resultant growth of bacteria and protozoa. Some of the non-cellular embryos which were isolated have survived longer; an eight-day embryo from a red half is shown in Fig. 19, consisting of granular protoplasm filled with large vacuoles and some pigment. The oldest embryo was still viable after a month (Photograph 43, following Photograph 10; and Fig. 20). This, from a yolk quarter, was a mass of protoplasm filled with yellowish granules and small vacuoles and larger spherical inclusions, possibly individual cells. There were fine strands radiating from the protoplasm to and possibly through the thin enveloping membrane characteristic also of normal free-swimming blastulæ; these are possibly short cilia, and the organism may have been slightly motile. Whether a non-nucleate egg can give rise to a true free-swimming ciliated blastula is still uncertain. It can, however, develop into a many-celled embryo having a certain amount of differentiation in that the cells are of different sizes; or into an embryo without cell boundaries but with differentiated protoplasm, perfectly viable a month after activation with parthenogenetic agents. The unfertilized and untreated sea-urchin eggs live only two or three days at room temperature, according to Loeb and Lewis (1902); they certainly do not survive longer than this in mass cultures without special care.

The striking similarity of the nucleate and non-nucleate eggs stops with the late blastula of one or two days (*Cf.* Photographs 35 and 40). I have seen no indication in the non-nucleate embryo of the formation of a gut or of the arms and skeleton characteristic of a pluteus. The normal fertilized egg has developed into a well-formed pluteus in three days, whereas the parthenogenetic merogone is still a spherical blastula. A photograph (41) of one of the 3-day parthenogenetic non-nucleate yolk quarters together with a pluteus of the same age from a normal fertilized egg shows the disparity in size and differentiation. The parthenogenetic yolk quarter of a month remained the same size with little change, whereas the normal pluteus in that time changes greatly in size and shape. Since the non-nucleate eggs are slower to develop in all stages, it is just possible that with longer time they might develop further than the blastula. Such a possibility is suggested by a comparison of larvæ from fertilized half and whole eggs 2 days old. In Photograph 42 (following Photograph 10) are shown normal plutei together with half-size plutei from fertilized white half-eggs. There are also present many spherical blastulæ of the half-eggs (which are slower to differentiate) not yet developed into plutei. As observation showed, many of these did develop into plutei later on. A comparison of Photographs 41 and 42 would at least suggest that the small blastula of the parthenogenetic merogone might also develop into a pluteus.

DISCUSSION

There has been considerable discussion as to whether cells without nuclei can divide, or whether asters can divide in the absence of chromatin. Boveri (1918) threw doubt himself on his earlier conclusions that asters (but not cells) could divide without chromatin. Dalcq (1931) has reviewed all the pertinent cases and has come to the conclusion that they are all subject to criticism, and that cell division does not take place without chromatin, and this seems to be the case with his own experiments. Fankhauser (1929, 1934) believes, however, that the large number of non-nucleate cells, some with amphiasters and even spindles, in his merogonic *Triton* eggs, indicates their origin from other non-nucleate cells, though he believes that nucleate cells must be also present to influence division. The presence of accessory sperm nuclei in the developing amphibian egg prevents these results from being absolutely clear-cut.

Wilson (1901) was the first to describe multiplication of asters in the entire absence of chromatin in the non-nucleate fragments (obtained by shaking) of *Toxopneustes* treated parthenogenetically, but he found no cell division. His results were questioned by Fry (1925), who maintained that in enucleate fragments accurately cut by hand and treated with parthenogenetic agents, asters might arise and irregular cleavage come in but the asters did not multiply. McClendon (1908), with a primitive micro-manipulator, very ingeniously removed the nucleus from the starfish egg and treated the egg with a parthenogenetic agent. He concluded that cells could divide without chromatin though the segmentation was quite irregular. These results were not unequivocal since many of the operated eggs contained chromatin.

There seems no doubt from the present observations that cell division can take place without nuclei. I have previously (1935) shown that the nucleus may be moved by centrifugal force from its normal position and cell division come in with no relation to its final position. Cleavage may also come in while the nucleus is still intact, as I noted previously in *Parechinus* (1935, Fig. 11), and as I have recently observed under certain conditions in *Arbacia*. The present work shows that the nucleus does not have to be present at all in cleavage. Asters arise *de novo* in non-nucleate eggs and become more numerous with time. Normal cleavages come in exactly as they do in nucleate eggs of the same sort and shape, and cleavage follows upon cleavage in a fairly orderly fashion.

An egg fragment lacking both maternal and paternal chromosomes has given rise by repeated cleavages to an embryo containing about 500 cells with a certain amount of differentiation. An embryo arising from a non-nucleate egg has lived a month; it is probable but not certain that these have cilia and are free-swimming. The early stages of development can, therefore, take place without chromosomes. This means that the maternal cytoplasm is of great importance and has within itself the potentialities of determining at least the early stages of development. These potentialities must either (1) be given to the cytoplasm by the nucleus previously, or (2) be innate in the maternal cytoplasm and entirely non-nuclear. They might be given to the cytoplasm either at the time of the breakdown of the germinal vesicle, or else earlier by the chromosomes of a preceding generation. The former alternative cannot be tested experimentally since the egg and egg fragments cannot be artificially activated, nor are they fertilizable, before rupture of the germinal vesicle. An influence on egg cytoplasm caused by the chromosomes of a preceding generation has been postulated for spiral twisting in snails and for certain characters in the silkworm and in corn. It would certainly be difficult to prove experimentally in the case of the sea-urchin egg. The second supposition, i.e., that the potentialities of early development are innate in the cytoplasm, restricts inheritance by genes to later developmental stages, and it may very well be that only the more specific and differential characters are controlled by the genes, whereas the general and fundamental characteristics of living matter are cytoplasmic. If further work shows, however, that the development of the parthenogenetic merogones does not stop with the blastula, but that they will develop into plutei, the results will be difficult to harmonize with the accepted ideas of the mechanism of genetical inheritance.

I have shown by previous studies (1932, 1933) that the visible granules in the eggs of several species of sea urchins are not of great importance in development. Any part of the egg seems capable of development, and many parts have, when fertilized, given rise to quite normal plutei with skeletons. Any difficulty in development seems to be mechanical rather than structural, such as the breaking apart of the blastomeres in the clear quarters of the *Arbacia* egg, owing to the thinness of the membranes; or the absence of cleavage planes in many of the red halves, owing to the very dense granular cytoplasm. No particular type of visible and moveable granules seems essential to development; these must be concerned with metabolism and respiration. It must therefore be the " ground substance " which is the material fundamental for development—the matrix, which is not moved by centrifugal force and which, in the living egg, is optically empty.

Summary

1. Non-nucleate fractions of *Arbacia* eggs obtained by centrifuging can be activated by parthenogenetic agents, and develop; these may be termed " parthenogenetic merogones."
2. Asters are formed and cell division takes place without nuclei.
3. Cleavages of non-nucleate fractions are strikingly similar to those of nucleate fractions (or whole eggs) of the same stage of development.
4. Many cleavages, in sequence, result in the formation of a blastula of as many as 500 cells.
5. Blastulæ emerge from the fertilization membranes; one embryo was still viable after a month.

LITERATURE CITED

Boveri, Th., 1918. Zwei Fehlerquellen bei Merogonieversuchen und die Entwicklungsfähigkeit merogonisher und partiell-merogonisher Seeigelbastarde *Arch. f. Entw.-mech.*, **44**: 417.

Dalcq, A., 1931. Contribution à l'analyse des fonctions nucléaires dans l'ontogénèse de la grenouille. I. *Arch. de Biol.*, **41**: 143.

Fankhauser, G., 1929. Ueber die Beteiligung kernloser Strahlungen (Cytaster) an der Furchung geschnürter Triton-Eier. *Rev. suisse de zool.*, **36**: 179

Fankhauser, G., 1934. Cytological studies on egg fragments of the salamander Triton. IV. *Jour. Exper. Zoöl.*, **67**: 349.

Fry, H. J., 1925. Asters in artificial parthenogenesis. II. *Jour. Exper. Zoöl.*, **43**: 49.

Harvey, E. B., 1932. The development of half and quarter eggs of Arbacia punctulata and of strongly centrifuged whole eggs. *Biol. Bull.*, **62**: 155.

Harvey, E. B., 1933. Development of the parts of sea urchin eggs separated by centrifugal force. *Biol. Bull.*, **64**: 125.

Harvey, E. B., 1935. The mitotic figure and cleavage plane in the egg of Parechinus microtuberculatus as influenced by centrifugal force. *Biol. Bull.*, **69**: 287.

HARVEY, E. N., 1931. The tension at the surface of marine eggs, especially those of the sea-urchin, Arbacia. *Biol. Bull.,* **61**: 273.

HARVEY, E. N., 1932. Physical and chemical constants of the egg of the sea-urchin, Arbacia punctulata. *Biol. Bull.,* **62**: 141.

LOEB, J., AND W. H. LEWIS, 1902. On the prolongation of the life of the unfertilized eggs of sea-urchins by potassium cyanide. *Am. Jour. Physiol.,* **6**: 305.

LUCKÉ, B., 1932. On osmotic behavior of living cell fragments. *Jour. Cell. and Comp. Physiol.,* **2**: 193.

MCCLENDON, J. F., 1908. The segmentation of eggs of Asterias forbesii deprived of chromatin. *Arch. f. Entw.-mech.,* **26**: 662.

SHAPIRO, H., 1935. The respiration of fragments obtained by centrifuging the egg of the sea-urchin, Arbacia punctulata. *Jour. Cell. and Comp. Physiol.,* **6**: 101.

WILSON, E. B., 1901. Experimental studies in cytology. I. *Arch. f. Entw.-mech.,* **12**: 559.

SEQUENCE OF FUNCTIONAL SEXUAL PHASES IN TEREDO

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In a recent number of this journal certain questions have been raised by Grave and Smith (1936) as to the sequence of the functional sexual phases and the proportions of individuals in each phase in the successive age groups of the variety of *Teredo navalis* found so abundantly at Woods Hole and other localities on the coast of southern New England. Evidence is now available which will answer these questions with some degree of positiveness.

In his earlier studies on the biology of this species at Woods Hole the senior author (Grave, 1928) stated that the females outnumber the males but he made no mention of the possibility of a change of sex. The recent investigations by Grave and Smith, however, indicate a sequence of alternating phases of functional sexuality comparable with that of certain species of oysters. They thereby confirm in most respects the conclusions reported during the past few years on the sexual rhythm of this species (Coe, 1933*b*, 1934*a*, 1935). Yet in regard to the proportions of individuals in each of the successive phases there is some divergence of opinion and it will be shown on the following pages that the reason for this discrepancy may be partly due to the failure of Grave and Smith to follow the life cycle through the entire sequence of sexual phases. Their published investigations cover only the two months from June 28 to August 27, 1935.

It is quite illogical to treat the sperm-producing and egg-producing phases of the teredo as distinct categories of individuals, since they represent merely successive stages in the life of a single individual. If the number of older animals in the functional female phase is found to be twice as great as in the alternate phase of sexuality, the explanation may be simply that the duration of one sexual phase averages twice as long as the other, always assuming that no selection, intentional or otherwise, as to ages is involved. Perhaps an exception should be made in regard to the relatively few individuals known as true males, in some of which the initial sexual phase may be retained for an indeterminate period. In still fewer cases the individual appears to be proterogynous.

Protandry in *Teredo* has been indicated, although not recognized as such, since the work of Quatrefages nearly a century ago, for he estimated that there were about twelve times as many females as males in the adult population. Nelson later suggested an even greater disparity of the sexes, with as many as five hundred females to one male among large individuals. Yonge (1926), on the other hand, found definite evidence of protandry in two specimens of *T. norvegica,* while Sigerfoos (1908) had previously suggested protandry in *Bankia gouldi.*

Substantial proof of a sequence of sperm-producing and egg-producing phases during the lifetime of the same individual has been published in the series of recent articles to which reference has been made (Coe, 1933*a*, 1933*b*, 1934*a*, 1935). In the first of these papers it was reported that an actual transition from the functional male phase to the functional female had been observed in about half of the small individuals infesting ropes in New Haven Harbor. The second paper (Coe, 1933*b*) gave detailed descriptions of the transformations of the primitive bisexual gonad of the very young individual, through the initial male phase and thence to the female phase toward the end of the breeding season. No evidence was available at that time that the female phase might again revert to the male condition but this transformation of sex was reported, with details of the cellular changes involved, in the third paper of the series (Coe, 1934*a*).

In a more recent brief summary of the sexual changes which the several types of individuals are believed to undergo the successive phases were schematized (Coe, 1935) as follows:

1. Male . . . female . . . recuperation . . . female.
2. Male . . . female . . . recuperation . . . male . . . female.
3. Male . . . recuperation . . . female.
4. Male ('true male') . . . recuperation . . . male . . . (female?).
5. (Exceptional.) Female . . . recuperation . . . (male?).

To this series the paper by Grave and Smith (1936) adds one additional aspect, namely, that the transformation from female to male phase may occur abruptly during the activities of the breeding season as well as in the recuperation period.

Proportion of Individuals in Each Functional Sexual Phase at Successive Ages and at Different Seasons

The accompanying Tables I and II indicate the sexual condition of 2,599 individuals grouped according to their sizes and approximate or maximum ages at each season of the year. The gonads of about three hundred of these were cut and mounted as serial sections; for each of the others the gonad was teased and examined microscopically. Most of the collections were made at Woods Hole, Massachusetts; others were obtained from Milford, Connecticut, through the courtesy of Dr. Victor Loosanoff, and a few from Barnegat Bay, New Jersey, through the kindness of Dr. T. C. Nelson.

Examination of 388 additional large individuals during the third week in June, 1936, showed 29 true males, 81 bisexual males, 17 hermaphrodites, 95 females with nearly ripe ova, and 166 females carrying embryos. Omitting the hermaphrodites, these numbers would represent a population having 29.65 per cent of the adults in the functional male phase, with 70.35 per cent functioning as females at the time of the examination. These data were obtained too late to be included either in the graph or in Table II, but they would not have changed greatly the percentages there indicated except for the larger proportion of females with embryos.

Since the sexual phases of most individuals change progressively with their advance in age, it is obviously impossible to classify them with great accuracy. Furthermore, there are so many individual differences in the expression of the male and female characteristics that the difficulty is greatly increased. Even if all individuals with ripe spermatozoa are classed as functional males, there are always found in a large collection some with a few sperm associated with many ova. Still more difficult and uncertain is the separation of functional males into the two groups of true males and bisexual (hermaphroditic) males, since all grades occur in the relative size and abundance of the ovocytes which even in many of the true males can be detected along the walls of some of the follicles.

Nor can the precise age of any individual be determined with accuracy because the teredo will not live and grow normally outside the wood in which it feeds. The maximum age is determined by reckoning from the time when the wooden block is placed in the water during the breeding season. The size is not strictly a reliable criterion of age, since rapidity of growth depends so largely on environmental conditions. The characteristics of the shell and of the pallets are sometimes helpful.

With these limitations in mind the data in Tables I and II will show the seasonal trend of the successive broods and the sequence of sexual changes which they undergo from immaturity to advanced age.

Attention may first be directed to the group of 43 individuals shown in Table I to have been collected on July 19 from a block which had been in the water for only 20 days. In this brief period 24, or more than half the total number, had already reached the functional male phase, while one only was producing ovocytes exclusively. The 18 individuals which were sexually immature were evidently still younger and later arrivals.

It thus appears that there were in this lot 24 protandric individuals as compared with one which was proterogynous. The evidence from the entire series of 911 small teredos listed in Table I is consistent with the conclusion that the first sexual phase is, with few exceptions, masculine.

The gonads of these young individuals, however, form an intergrading series, from those which have only a few small ovogonia or ovocytes on the walls of the follicles (true males) to those which are so strongly feminine that they consist mainly of large ovocytes, with relatively few spermatogenic cells in the lumens. All except the true males are grouped in Tables I and II as bisexual males.

It will be noted that more than 10 per cent of the small teredos listed were in the female phase. Presumably most of these were dwarfed animals that had already completed a brief initial male phase rather than strictly proterogynous individuals.

The second sexual phase, which immediately follows the initial male phase except in the autumn, is usually feminine. True males, however, may resume and continue spermatogenesis for an indeterminate period.

No proof is at present available that a third sexual phase occurs in these localities during the same year. More frequently a long recuperation period follows the completion of the female phase in late summer and autumn. During the winter the gonad of such individuals is reorganized and the third sexual phase, which may be of either type of sexuality, develops during the late winter and early spring. The breeding period lasts from about the middle of May to the middle of October.

The over-wintering teredos will thus include individuals of all ages, from those young ones which settled on the blocks in the autumn and are still sexually immature to those which completed at least two sexual phases the preceding breeding season. The most numerous group, however, will be of the younger ages.

In the pre-spawning season (Table II) the two alternate sexual phases are found in about equal numbers, or with a small majority in the female phase. Some of the individuals classed as being in the bisexual male phase evidently have but a brief period of sperm production at the beginning of the second spawning season, for the ratio of animals in the female phase appears to increase with advancing age (Table II). The percentages in each sexual phase are shown graphically in Fig. 1. It is evident either that the number of animals changing from male to female phase is greater than the number going in the opposite direction or that the female phase is of longer duration than the alternative aspect of sexuality.

The data compiled by Grave and Smith (1936), on the other hand, would indicate that the ratio of animals in the female phase decreases during the summer from approximately 3 to 1 (150–58) in June to 46–55 (65?) in August, or about 1 to 1 (58–55) if the hermaphrodites are included among the females. The differences between these data and those here recorded may be due in part to chance selection and in part to differences in classification. In many cases the bisexual gonad shows such an intermediate condition between male and female phases as to make any distinction more or less arbitrary. Moreover, shortly after the middle of August the first young of the year may attain a length of 100 mm. or more—a size sufficient to be classed as "large specimens." If some of these were to be included with the older individuals there would appear to be a rapid increase in the male-phase ratio.

TABLE I

Sexual phases of small individuals (20–60 mm. in length) of approximately known ages

Date	Maximum age	Sexually immature	True male phase	Bisexual male phase	Young female phase	Mature female phase	Females with embryos	Total
	days							
Mar. 29	—	0	4	18	0	0	0	22
May 19	—	0	5	21	20	0	0	46
June 25	—	0	5	29	13	7	0	54
July 19	20*	18	6	18	1	0	0	43
Aug. 18–23	71	8	71	415	6	4	3	507
Sept. 19–24	112	0	5	80	16	12	6	119
Sept. 30	42	41	0	12	1	0	0	54
Nov. 1	73	16	11	32†	5	2	0	66
Total		83	107	625	62	25	9	911
Per cent		9.11	11.75	68.61	6.80	2.74	0.99	

* From Barnegat Bay, N. J. Courtesy Dr. T. C. Nelson.

† Winter condition.

It is, however, in the late spawning season that the greatest preponderance of large individuals in the female phase is found; for instead of the former ratio of about 2 to 1, a collection of 164 large individuals in September gave a female ratio of approximately 10 to 1 (not counting 7 hermaphrodites), as shown in Table II and Fig. 1. These animals, although only 3 to 4 months of age, had already reached a length of more than 100 mm. and had, with relatively few exceptions, already completed the initial male phase. By selecting a group measuring 70–95 mm. in length, some of the younger, male phases were included and the ratio approximated equality (Fig. 1). Selection of 119 still smaller and younger animals on the same dates showed more

TABLE II

Sexual phases of large individuals (90–275 mm. in length)

Date	Maximum age, months	True male phase	Bisexual male phase	Hermaphrodites	Unripe female phase	Mature female phase	Females with embryos	Spawned out	Total
	Pre-spawning Season								
Apr. 20–25....	7–10	11	66	0	48	81	0	0	206
29.......	7–10	0	33	0	16	0	0	0	49
30.......	7–10	3	21	0	30	0	0	0	54
May 10.......	8–11	8	32	0	5	9	1	0	55
Total.........		22	152	0	99	90	1	0	364
Per cent.......		6.05	41.76	0	27.16	24.73	0.3	0	
	Early Spawning Season								
May 19.......	8–11	7	35	0	10	11	11	0	74
25.......	8–11	4	35	0	11	14	12	0	76
June 7–13....	9–12	11	12	0	32	57	17	0	129
16.......	9–12	3	18	0	7	24	6	0	58
24–26....	9–12	7	39	0	18	51	11	0	126
Total.........		32	139	0	78	157	57	0	463
Per cent.......		6.91	30.03	0	16.85	33.90	12.31	0	
	Middle Spawning Season								
July 23.......	12–14	5	1	0	0	5	22	0	33
Aug. 18–21....	2–3	18	60	3	0	82	57	0	220
Total.........		23	61	3	0	87	79	0	253
Per cent.......		9.09	24.1	1.19	0	34.40	31.20	0	
	Late Spawning Season								
Sept. 19–24....	3–4	9	5	7	0	52	84	7	164
Per cent.......		5.5	3.0	4.3	0	31.7	51.2	4.3	
	End of Spawning Season								
Oct. 3–6.....	4	2	5	0	4	13	0	94	118
7–8.....	4	8	11	0	0	23	9	41	92
Nov. 1.......	2–3	0	0	0	0	6	0	73*	79
13.......	3	0	0	0	0	0	0	65*	65
15–17....	5–6	3	17	0	0	0	0	70*	90
Total.........		13	33	0	4	42	9	343	444
Per cent.......		2.92	7.42	0	0.90	9.45	2.03	77.25	
Total all seasons......		99	390	10	181	428	230	350	1688
Per cent.............		5.86	23.10	0.59	10.72	25.35	13.62	20.74	

* Winter condition.

than twice as many functioning as males as were in the alternate phase of sexuality, and the proportion of sperm-bearing individuals in the total population was still greater, since the number of young animals greatly exceeded that of the older individuals in the female phases.

Toward the end of the breeding season, when most of the older individuals have spawned out there is again an approximately equal number of the large animals in each sexual phase (Table II; Fig. 1). Incom-

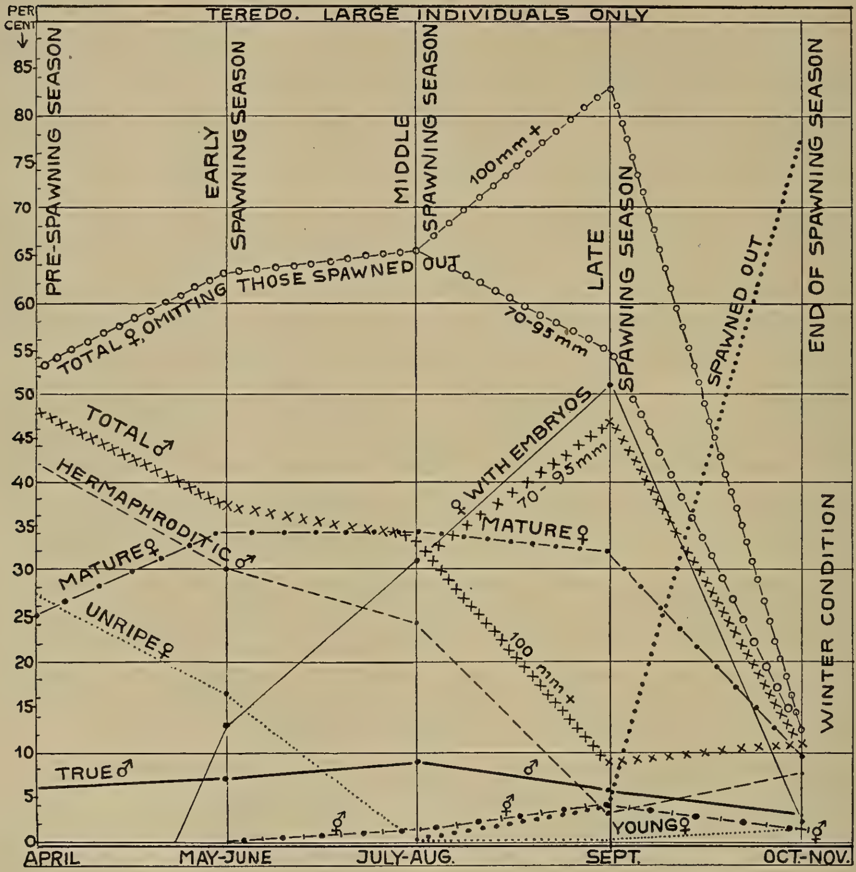

Fig. 1. Graphs showing the proportions of large individuals (90–275 mm. in length) in each phase of sexuality before, during and at the end of the breeding season. Based on a total of 1,688 individuals. It is to be noted that the chart includes two generations of animals, the over-wintering individuals being supplanted by the new broods which attain the "large" size after the middle of the spawning season. The graphs indicating total male and female phases in the late spawning season differentiate two classes of individuals, namely, those 70–95 mm. in length and those of still larger sizes.

pleted sexual phases may be retained during the winter, but most of the partially developed sexual cells will be absorbed by phagocytosis.

The Normal Life Cycle

Turning now from the statistical study of selected groups of animals to a consideration of the sequence of sexual phases realized during the normal lifetime of the single individual, it becomes evident that several different influences are involved. Examination of the gonads of animals of approximately known ages shows clearly the protandric nature of the vast majority of all individuals. Ripe spermatozoa may be produced within three to five weeks after metamorphosis.

The ensuing phase is predominantly female, the percentage of females being influenced by the season when hatched and presumably by hereditary factors as well as by the environment, since all grades of bisexuality appear simultaneously. Eggs may have been liberated and embryos found in the gill chambers within six weeks after metamorphosis. There is no doubt of the strong tendency toward a series of alternating phases, the number of such phases that may be realized by any individual depending largely upon its length of life. At least four alternating phases may occur—two in each of two breeding seasons, but it is not improbable that the number may occasionally exceed four.

The cooler water of early autumn checks the reproductive activity of such individuals as have not already ceased gametogenesis for the season and entered upon the recuperative phase. Most of the oldest individuals are killed by parasitic Protozoa about the middle of the breeding season, as noted by Grave and Smith (1936). The epidemic later affects the earlier broods of the same year, causing the death of many individuals not more than three to five months of age.

The gonads of the survivors, including the spawned-out individuals as well as the others, are reorganized during the winter, in many cases with a change in the sexual phase. Disintegrating residual cells from the previous phase of sexual activity are removed by phagocytosis before the proliferation of the definitive germinal cells.

True Male Phase

This phase represents the extreme aspect of masculinity in that the gonads have only a very few ovogonia or minute ovocytes along the walls of the follicles. Such individuals form but a small proportion of the youngest sexually mature animals (Table I). They occur also as the third sexual phase, following the discharge of the ovocytes of the female phase, but appear to be indistinguishable from those which retain the initial male phase after a long period of growth (Table II).

Individuals of this type were observed by Grave and Smith (1936), who conclude that a "careful study of adult individuals leads us to doubt if there is such a thing as a true male or a true female *Teredo* in the sense that it remains permanently one sex, as postulated by Coe, who agrees that sex inversion in true males may ultimately take place." The two principal parts of this sentence are obviously contradictory, and the word "permanently" has been substituted for "indefinitely" which was used in the papers under discussion. Otherwise there would have been no ground for disagreement, for "true males" are defined in those papers (Coe, 1933*b*) as individuals in which "the preliminary male phase is of longer duration and the crop of spermatozoa is much larger."

The fact that such individuals are found at earliest sexual maturity as well as later is indicative of the operation of genetic factors which differentiate them from others of the same brood. The latter reveal by their gonads a more feminine tendency and are classed as bisexual males or protandric females. This appears to be equally true of other mollusks in which a similar type of sexuality has been reported (Coe, 1934*b*, 1936).

Bisexual Male Phase

The majority of young individuals on reaching sexual maturity are obviously protandric, with gonads showing a highly variable proportion of spermatogenic and ovogenic cells. During the recuperation period there is frequently a return to a similar condition (Tables I and II, Fig. 1).

Functional Bisexuality

Most of the sexually mature teredos examined during the breeding season were actively producing either sperm or ova, but a few individuals occur, as Grave and Smith (1936) have reported, which contain functional gametes of both types. Only 10 of these hermaphrodites were recognized among the 2,599 animals examined (Table II).

In other cases spermatogenesis is well advanced while embryos are present in the gill chambers. Moreover, in an occasional individual some follicles of the gonad may be producing spermatozoa while adjacent follicles contain nearly ripe ova. Such hermaphrodites would not of themselves be indicative of a change of sex in either direction. Of the 618 sexually active individuals reported by Grave and Smith, 27 were classed as hermaphrodites. A collection of 388 adults from the Eel Pond at Woods Hole examined during the third week in June, 1936, contained 17 individuals in this phase of sexuality.

PROTEROGYNY

The finding of an occasional individual in the female phase at earliest sexual maturity indicates that the initial male phase may sometimes be aborted or omitted, as it is known to be in some other species of normally protandric mollusks (Coe, 1936).

POST-SPAWNING PHASE

The " spawned-out " condition which may lead again to the bisexual phase or to either of the unisexual phases during the period of recuperation appears to follow the female phase more often than otherwise, although a similar condition results when spermatogenesis ceases and the sperm are discharged without a reciprocal growth of ovocytes. This phase occurs principally toward the end of the breeding season (Table II, Fig. 1), but is also found whenever gametogenesis ceases from any cause.

SUMMARY

Examination of the gonads of 2,987 specimens of *Teredo navalis* of all ages, at all seasons, and during three successive years supplements the conclusions previously published regarding the sequence of sexual phases in this species. The functional phase, if any, in which any given individual is found will, in general, depend upon: *a,* age; *b,* size; *c,* season; *d,* season when hatched; *e,* its particular combination of genetic factors.

Each normal individual shows a tendency toward an alternating series of functional sexual phases. There is good evidence that at least four of these phases may sometimes be completed in a lifetime of less than two years, although the average individual experiences hardly more than one or two.

Among the variable sequences of phases which different individuals may experience the following may be considered typical:

1. Male . . . female.
2. Male . . . female . . . male . . . female.
3. Male . . . female . . . male.
4. Male . . . female . . . female.
5. Male (" true male ") . . . male . . . female.
6. Male (" true male ") . . . male.
7. Female (exceptional) . . . male . . . female.

Because the population consists of a vastly greater number of individuals of the younger ages than of the older age groups, it is obvious that death by suffocation, starvation, parasitization or some other cause will usually terminate the individual's existence before the normal cycle is completed.

Protandric bisexuality is generally characteristic of the early sexual phases, with a tendency toward alternating unisexuality in later life. Of the latter, the female phase is usually of longer duration than the alternate male phase.

The proportion of individuals in each sexual phase in the general population or in selected groups will obviously be correlated with a combination of the conditions mentioned above, but limited by associated environmental influences. The total population of teredos living in a particular block of wood may thus consist of upwards of 90 per cent of individuals in either functional sexual phase or of about equal percentages of each, according to the age group or groups represented at the time of the examination.

Attention has been previously (Coe, 1934*a*) called to the similarity of the sexual rhythm in *Teredo* to that of the larviparous oysters, " although the usually shorter life of the former limits the number of the alternating sexual phases." There is some resemblance also to the sexual changes in the oviparous oysters (Coe, 1934*b*) and in the hard-shell clam *Venus* (Loosanoff, 1936), which are predominantly, but not exclusively, protandric, with a later change to the seasonally unisexual condition of the older individuals, in which there is an approximate equality in the sex ratio.

LITERATURE CITED

Coe, W. R., 1933*a*. Destruction of mooring ropes by Teredo; growth and habits in an unusual environment. *Science,* **77**: 447.

Coe, W. R., 1933*b*. Sexual phases in Teredo. *Biol. Bull.,* **65**: 283.

Coe, W. R., 1934*a*. Sexual rhythm in the pelecypod mollusk Teredo. *Science,* **80**: 192.

Coe, W. R., 1934*b*. Alternation of sexuality in oysters. *Am. Nat.,* **68**: 236.

Coe, W. R., 1935. Sequence of sexual phases in Teredo, Ostrea and Crepidula. *Anat. Rec.,* **64**, suppl.: 81.

Coe, W. R., 1936. Sex ratios and sex changes in mollusks. Manifestation Paul Pelseneer. Bruxelles, 1936: 69.

Grave, B. H., 1928. Natural history of shipworm, Teredo navalis, at Woods Hole, Massachusetts. *Biol. Bull.,* **55**: 260.

Grave, B. H. and Jay Smith, 1936. Sex inversion in Teredo navalis and its relation to sex ratios. *Biol. Bull.,* **70**: 332.

Loosanoff, Victor, 1936. Sexual phases in the quohog (Venus mercenaria). *Science,* **83**: 287.

Sigerfoos, C. P., 1908. Natural history, organization and late development of the Teredinidæ, or ship-worms. *Bull. U. S. Bur. Fish.,* **27**: 193.

Yonge, C. M., 1926. Protandry in Teredo norvegica. *Quart. Jour. Micr. Sci.,* **70**: 391.

THE OCCURRENCE AND SIGNIFICANCE OF NITRITE IN THE SEA

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In studying the nitrogen cycle in the sea a consideration of the nitrite concentration in the water is especially useful, because of its intermediate position between ammonia on the one hand and nitrate on the other. Although it is not a stable end-product, its very instability makes it a possible indicator of the state of equilibrium between the oxidation and reduction processes making up the cycle. Its order of magnitude in the water is much smaller than that of nitrate, or even ammonia, but the methods for its analysis are correspondingly more accurate, so that its normal variations may be followed as closely as those of the other nitrogen compounds.

In view of the ease with which nitrite determinations may be made, it is surprising that there are not more data on its occurrence in sea water. Raben (1914, 1918) and Brandt (1927) were among the first to report such figures, but their data were only scattered and fragmentary. Orr (1926) reported some analyses of samples from the vicinity of the Clyde Sea, made observations on diatom cultures, and discussed the factors concerned in the appearance and disappearance of nitrite. Harvey (1926) failed to find significant amounts of nitrite in the English Channel or in deeper waters of the Atlantic further south, but noticed their occasional appearance in stored samples. Atkins (1930), however, was able to make a fairly comprehensive study of the seasonal changes in nitrite in the English Channel, finding high concentrations at certain levels and at certain times. Orton (1924), investigating various influences upon the growth of oysters, found moderately high concentrations in samples from Plymouth Sound, Thames Estuary and Helford River. He also followed nitrite changes in the water of storage tanks.

In a few cases, figures for nitrite have been reported incidentally, or in connection with other chemical studies of sea water, as by Braarud and Klem (1931), Robinson and Wirth (1934), Rakestraw (1932, 1933), Matsudaira and Yasui (1932, 1933), and in connection with the Antarctic cruises of the "Discovery" (1932). Three good seasonal studies have appeared more recently, by Soot-Ryen (1932), Verjbinskaya (1932), and Cooper (1933). The last of these is most comprehensive, containing very complete data for the relationship between various chemical factors in the water. Unfortunately, its limitation to one or two shallow stations in the English Channel raises a question as to the validity of its conclusions when applied to other, more general conditions.

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[1] Contribution No. 110.

The present paper includes results collected over a period of five years at the Woods Hole Oceanographic Institution in the effort to explain the production and utilization of nitrite and its place in the nitrogen cycle of the sea and the general process of marine metabolism. The data include analyses from:

1. A small group of inshore stations in shallow bays.
2. Stations on the continental shelf to the south of Cape Cod, including two serial sections to the edge of the shelf.
3. A number of stations in the Gulf of Maine, so grouped as to indicate seasonal variations for more than a year, at seven different locations.
4. A group of deep sea stations to the south of Cape Cod, from the edge of the continental shelf to Bermuda, at different times of the year.
5. Stations to the south of Bermuda, across the edge of the Sargasso Sea to the Bahamas, and fifteen stations in the Caribbean.
6. A large number of scattered surface samples, at various places and times.
7. Bottom muds and waters contained in them.
8. Samples stored under a variety of conditions.

Methods

The Griess-Ilosvay method, used in this work as in that of practically all previous investigators, has been repeatedly described, with minor variations. It depends upon the color developed in the sample by means of sulphanilic acid and alpha-naphthylamine (or its dimethyl or diethyl derivative). Buch (1923) established its suitability for sea water by a careful spectrophotometric study. It is simple, reliable and not easily susceptible to temperature changes and other fortuitous factors. There is no salt effect upon the color development, so that standards may be made up in distilled water. It was easily established that the color developed was constant between 2 and 48 hours, an adequate range for routine purposes.

Two milliliters of sulphanilic acid solution (8 grams in 1 liter of

30 per cent acetic acid) are added to 100 ml. of the sample, followed by 2 ml. of a solution of alpha-naphthylamine, or diethyl-alpha-naphthylamine (8 grams in 1 liter of 30 per cent acetic acid, with 10 ml. of conc. HCl added). After two hours or more the samples are compared with appropriate standards. This was done in a colorimeter of the Hehner tube type, which has long been used in this laboratory for phosphate, nitrite and other determinations.

We have seldom had trouble in obtaining nitrite-free distilled water (as Cooper seems to have had), and have very frequently found sea water samples free of nitrite to the limit of sensitivity of the method, which is well under .005 microgram atom per liter. Stock standards have been made either from sodium nitrite or from silver nitrite, precipitated by NaCl. In either case, such solutions may be preserved for many weeks and their concentrations checked by titration with $KMnO_4$, or iodimetrically. Dilute standards for use are prepared from these and kept for not longer than one day.

TABLE I

Accuracy of the analytical method for nitrite.

Depth in meters	Triplicate analyses			
	A	B	C	
30	4.3 .307	4.3 .307	4.4 .315	mg./cu. m. µg. at./liter
40	1.9 .135	1.9 .135	1.9 .135	mg./cu. m. µg. at./liter
50	1.6 .115	1.6 .115	1.65 .118	mg./cu. m. µg. at./liter

Throughout this work results will be expressed in microgram atoms per liter, according to the recent recommendations of the International Council. These may be translated into the older units of milligrams of nitrite-nitrogen per cubic meter by multiplying by 14.

In order to indicate the accuracy and reproducibility of results by this method there are given in Table I the results of triplicate analyses (A, B, C) on three different water samples, expressed in both the old and new systems of units. Later results are given to the nearest .005 microgram atom.

Variability of Nitrite

As far as our experience goes, the nitrite concentration in sea water varies normally from 0 to .35 microgram atom per liter. Occasional values up to double this amount have been encountered, and the highest found (in Cape Cod Bay) was .91. No values like those reported by Atkins (2.78, or 38.9 mgm./m.^{3}), or by Matsudaira and Yasui, in the Yellow Sea, have ever been found in normal waters on this side of the Atlantic.

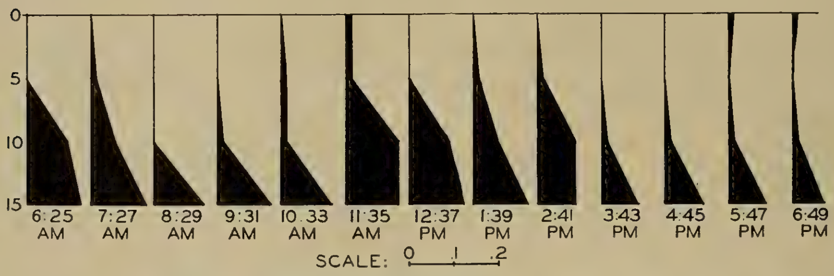


Fig. 1. Variation of nitrite during a tidal cycle. Station K–2, Buzzards Bay. Ordinates, depths in meters.

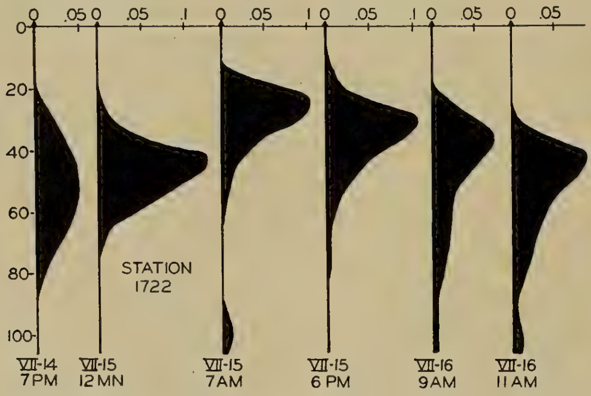


Fig. 2. Variation of nitrite during a tidal cycle. Station 1722, Gulf of Maine. Ordinates, depths in meters.

Nitrite is one of the most variable factors in the sea. Analyses from two stations close together, or from the same station at different times not far apart, sometimes yield quite different results. In fact, the horizontal distribution of nitrite is highly dependent upon local conditions. This would be better appreciated if previous data had not been so scattered and incomplete. Cooper seems to have been the only one sufficiently well aware of the fact.

In view of this, it is rather surprising that any general trends can be seen and conclusions drawn. Absolute values for nitrite concentration, especially isolated ones, are of little use; only by a comparison of relative amounts, with fairly complete data, can one obtain a clear picture of conditions.

To show how general trends do appear, however, the nitrite concentration was followed at two stations (K–2 and 1722) throughout a complete tidal cycle. The results are shown in Figs. 1 and 2. Here, as in all the subsequent figures, the vertical distribution of nitrite is represented by a solid black area.

The vertical variation in nitrite concentration is one of the most striking and interesting facts concerning it. As will later be shown, both the production and utilization of nitrite are processes taking place in particular, but different, layers. This often gives rise to large vertical differences, and usually, in the summer, a maximum zone, of variable depth, sharpness and intensity. Good examples of this are found in Atkins' results from the English Channel. Strangely, Cooper's data from the same location, a few years later, fail to show the phenomenon —probably, as he suggests, due to different hydrographic conditions that year.

Table II

Stations in shallow bays. Nitrite concentrations in microgram atoms per liter.

Station:	K-1	K-2	K-3	L-1		L-2		R-3	R-4	S-1	S-2
Depth in meters	8-30 1931	8-30 1931	8-30 1931	7-18 1931	8-28 1931	7-18 1931	8-28 1931	8-26 1932	8-26 1932	7-14 1932	7-14 1932
0	0	0	0	0	0	0	.07	0	0	0	0
5	0					0	.01	0	.41		
10	.01	0	0	0	.01	0	.10	.085		.61	.86
15		.01									
20			.01	0	.01	0	.085		.43		.20
40										.91	

The nitrite concentration in bottom muds is most highly variable and will receive special attention later.

All this variability in nitrite concentration is only to be expected from the precarious position which it occupies in relation to other substances in the water. Conceivably, it may be produced by oxidation of ammonia or reduction of nitrate, by means of bacteria, catalytically or photochemically. It is not impossible that the phytoplankton may play a part in the oxidation or reduction. In any event, nitrite is one step in the process of decay and "mineralization" of organic matter, either formed or particulate, or dissolved in the water.

On the other hand, nitrite may be destroyed by: (1) oxidation to nitrate or reduction to ammonia—atmospherically, photochemically, or

TABLE III

Stations on the continental shelf, near Cape Cod. Nitrite concentrations in microgram atoms per liter.

Station:	1270	1295	1271	1296	1272	1297	1273	1298	1260	1299
Depth in meters	6-29 1932	7-20 1932	6-29 1932	7-20 1932	6-29 1932	7-20 1932	6-29 1932	7-21 1932	6-28 1932	7-21 1932
0	0	—	.06	—	.01	.05	.07	—	—	—
10	0	0	0	0	0	0	.01	0	0	.01
20	.01	0	0	0	0	.01	.01	0	0	.01
30		.01		.115		.235		.01		0
40		.085	.07		.14		0			
50								.41		
60						.09			.23	.19
75					0		.26	.36		
100										
125									0	.01
150										
175										.01

Station:	1261	1283	1282	1281	1280	1725	1739	1684	2143	2156
Depth in meters	6-28 1932	7-1 1932	7-1 1932	7-1 1932	7-1 1932	7-25 1933	7-31 1933	7-27 1933	5-8 1934	5-11 1934
0	0	0	0	0	0	0	0	0	.04	0
10		0	0	0	0	0	0	0	.05	0
20	0	0	0		0	0	.02	0	.06	0
30		.17	0	0			.44	.01	.07	0
40		.065				.44	.39	.01		
50			.06	.33	0		.36	.12	.08	.085
60						.20	.24	.03		
75							.36	0	.12	.11
100	.01			.20	.07	.02		0	.18	.27
125								.01	.26	.26
150						.03		.05	.17	.07
175					.05				.05	.08
200	0									.01

by bacteria; or, (2), consumption by phytoplankton. We cannot be altogether certain whether or not free nitrogen enters into the process in any way. However, at any moment, the concentration of nitrite is an expression of the equilibrium between production and destruction.

The distribution of nitrite may therefore be related to a large number of factors: the distribution of bacteria, phytoplankton and zoöplankton; temperature; light intensity; oxygen tension in the water; density and circulation of the water; and perhaps other seasonal influences.

TABLE IV

Serial sections on the continental shelf. Nitrite concentrations in microgram atoms per liter.

Line "A"
July–August, 1931

Depth in meters	Miles off:									
	1	2	5	7	10	13	39	61	82	90
0	0	0	0	0	0	0	0	0	0	0
10	.07			0	0	0	0	.09	0	
25		.13	.06	.04		.10			0	0
35					.03	.115				
50									0	0
65							.40			
100								.135		0
565									.035	
885										0

Line "D"
August 5–6, 1931

Depth in meters	Miles off:								
	1	2	5	10	20	30	40	50	66
0	0	0	0	0	0	0	0	0	0
10			0						
20	.05	.01		0	.01	0	0	0	0
40			.09	.215					
60					.66				0
75						.05	.15	.165	
100									0
200									.035

DESCRIPTION OF DATA

The results from shallow, inshore stations, shown in Table II, were mostly obtained in the early stages of the work and are useful in demonstrating that the occurrence of nitrite is not closely associated with such influences as land drainage. Although concentrations are usually low, there are exceptions, as at Stations S–1 and S–2, where the highest concentrations of all were found. Great differences in local conditions evidently exist.

The stations on the continental shelf, shown in Tables III and IV, and in Fig. 3, present a fairly regular picture. Since these were all sampled during the summer months, nitrite was seldom found at the surface in more than small traces, while significant amounts were encountered below 30 meters, or near the bottom, if shallower than that. Surface conditions off New York Harbor were somewhat exceptional. Lines *A* and *D,* to the edge of the shelf, bring out the fact that the rate of nitrification on the bottom, as indicated by nitrite concentration there, is apparently greatest at depths of 50 to 75 meters. The numbering of stations on these lines (*A*–5, *A*–39, *D*–20, etc.) indicates the number of miles off-shore, in each case. An unfortunate spacing of the sampling levels at the deeper stations on these lines, as well as elsewhere on the shelf, failed to reveal the zone of maximum nitrite generally found at such stations between 30 and 50 meters, and which probably existed here also. At the deepest stations, along the edge of the shelf (1725, 1684, 2143 and 2156), this maximum zone is clearly seen. Its depth varies considerably, showing the influence of the adjacent oceanic water, in which the maximum zone is invariably deeper than at stations on the shelf.

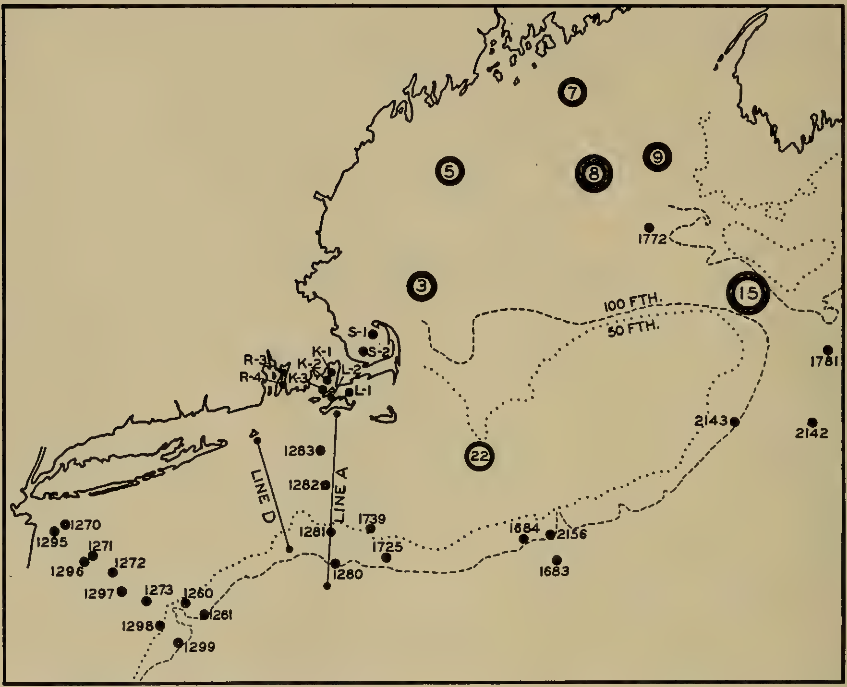

7
5
8
9
1772
3
100 FTH.
50 FTH.
15
1781
S-1
S-2
K-1
K-2
K-3
L-2
L-1
R-3
R-4
2143
2142
22
1283
1282
LINE D
LINE A
1739
1281
1684
2156
1725
1280
1683
1270
1295
1271
1296
1272
1297
1273
1260
1261
1298
1299

FIG. 3. Location of stations in the Gulf of Maine and vicinity.

TABLE V

Deep sea stations, Cape Cod to Bermuda. Nitrite concentrations in microgram atoms per liter.

	Group 1			Group 2				Group 3					Group 4		Miscellaneous							
Station:	1731	2313	2455	1732	2315	2456	2457	1462	1734	2316	2458	2460	1465	2317	1463	1640	1683	1730	1781	2142	2450	2451
Depth in meters	7-26 1933	1-21 1935	7-22 1935	7-27 1933	1-21 1935	7-22 1935	7-23 1935	2-7 1933	7-28 1933	1-23 1935	7-23 1935	7-24 1935	2-10 1933	1-23 1935	2-7 1933	5-17 1933	7-27 1933	7-27 1933	9-10 1933	5-7 1934	7-21 1935	7-21 1935
0	0	.02	0	0	.08	0	.01	—	0	.065	0	0	.16	.065	—	.10	0	0	0	0	0	0
10	0		0					.14					.01		.035		0	0	0	0		
20	0	.06	0	0	.15	0	0	.14	0	.065	0	0	0	.06	.01	0	0	0	0	.02	0	0
30	0			0									0		.01	0	0	0	0			
40	0							.15					.03				0	0	0	.12		
50	0	.30	.33	0	0	.215	.30	.135	0	.06	0	0	0	.03	.01	0	.08	0	.18		0	0
60	0			0				.15	0								.06	.03	.165	.38		
75	.20	.06		0	.16				.01	.05	0	0		0	.03		.01	.02	.05	.60		
100	.065	.04	0	0	.32	.14	.06	.21	.05	.06	0	0		0	.03	.06	.01	.02	.02	.51	0	0
150	.01	0		.05	.14			.185	.02	.065	0	0	0	.065	.035	.07	.02	.01	.02	.29	0	0
200	.01	0	0	0	.08	0	0	.185		.06	0	0	.03	.035	.01	.04	.03	.01	.02		0	0
250		0			.08				0	.065		0					.03		0	.01	0	0
300		0	0	.065	0	0	0	.01	0	.065	0	0	0	.06	0	.04	.02		0	0	0	0
400	.01		0	.01	0	0	0	.165	0	0	0	0	0	0	0	.01	.02	.01	.01	.01	0	.01
500			0			0	0	.07			0	0	.02	0	0	0	0		.01	0	0	0
600	0		0	.01	0			.01	.02	0	0	0	.03	0	0	0	0	.01	0	.02	0	0
800	0			0	0			.01	0	0	0	0	0		0	0	0	.06	0	0	0	0
1000	0			0	0			0		0	0	0	0		0	0	0	.01	.01	0	0	0
1100															0		0					
1200	0				0			.01			0	0	0		.01		0	0	.01		0	0
1400	0							0					0		0		0	0	.02			
1600	0							0					0		0			0	.02		0	0
1800													0						.01	0		
2000								0					0		0			0	.03	.02	0	0
2500													0		0						.035	.01
3000													0		.035							
3500															.04							
4000															0		0					

TABLE VI

Seasonal variation of nitrite in the Gulf of Maine. Nitrite concentrations in microgram atoms per liter.

Location 3

Station:	1284	XX	1699	1748	1857	1864	1909	2022	2074	2168	2220	2274
Depth in meters	7-12 1932	5-6 1933	7-3 1933	9-4 1933	10-19 1933	12-2 1933	1-9 1934	3-22 1934	4-18 1934	5-22 1934	6-26 1934	9-19 1934
0	0	0	0	0	.215	.09	.08	.085	.10	.01	.035	0
10	0	0	0	0		.08	.02	.085	.10	0		0
20	0	.06	0	0		.08			.115	0	.02	
30	0	.17	.01	0	.28	.08	.03	.11	.135	0	0	.11
40	.165		.14	.04						.22	.18	
50	.32	.20	.11		.21	.07	0	.085	.135		.135	0
60	.32		.07	.02		.065				.39	.09	0
80			.01	0	0	0	0	.09	.06	.14	.04	0
100	.02	.02	0	.03	.01	0	0	.085	.02	.115	.02	0
125			0	0				.065	0	.10	.02	0
150	0		.01	0	.01	0	.01	.04	0	.01	.03	0
175								.03	0	0	.01	0
200	.035		0	0	0	0	0		0	0	.02	0
225		0						.03	0	0		
250			0	0	0		0					0

Location 5

Station:	1287	1705	1761	1871	1911	2031	2083	2176	2224	2282
Depth in meters	7-10 1932	7-7 1933	9-6 1933	12-3 1933	1-9 1934	3-23 1934	4-19 1934	5-24 1934	6-28 1934	9-20 1934
0	.035	0	0	.035	0	.09	.11	.08	0	0
10		0	0	.035	0	.085	.115	0	0	0
20	0	0	.08	.02	0		.12	.18	0	
30	.10	.07	0	.02	0	.085	.115	.21	.28	.035
40	.25	.085	0	.02	0			.21	.235	
50	.09	.09	0	.02	0	.08	.12	.215	.215	0
60	.03	.04	0	.03	0			.185	.15	
80	.01	.01	0	.02	0	.05	.05	.115	.15	0
100	.01	.01	0	.01	0	.035	.035	.085	0	0
125	.01	.01	0	.01	0	.02	.03	.10	0	.01
150	.03	.01	0	.03	0	.03	.01	.05	0	0
175		.01	0		0	.035	0	0	0	0
200					0				0	

Table V contains the deep stations from Cape Cod to Bermuda. A number of these are located in roughly corresponding positions and are arranged in four groups accordingly. Of these, No. 3 is most complete, with four stations at different times of the year. These have been plotted in Fig. 8 to show the seasonal variations. At all these stations, the maximum zone is found between 50 and 100 meters, with only traces

TABLE VII

Seasonal variation in the Gulf of Maine (continued). Nitrite concentrations in microgram atoms per liter.

Location 7

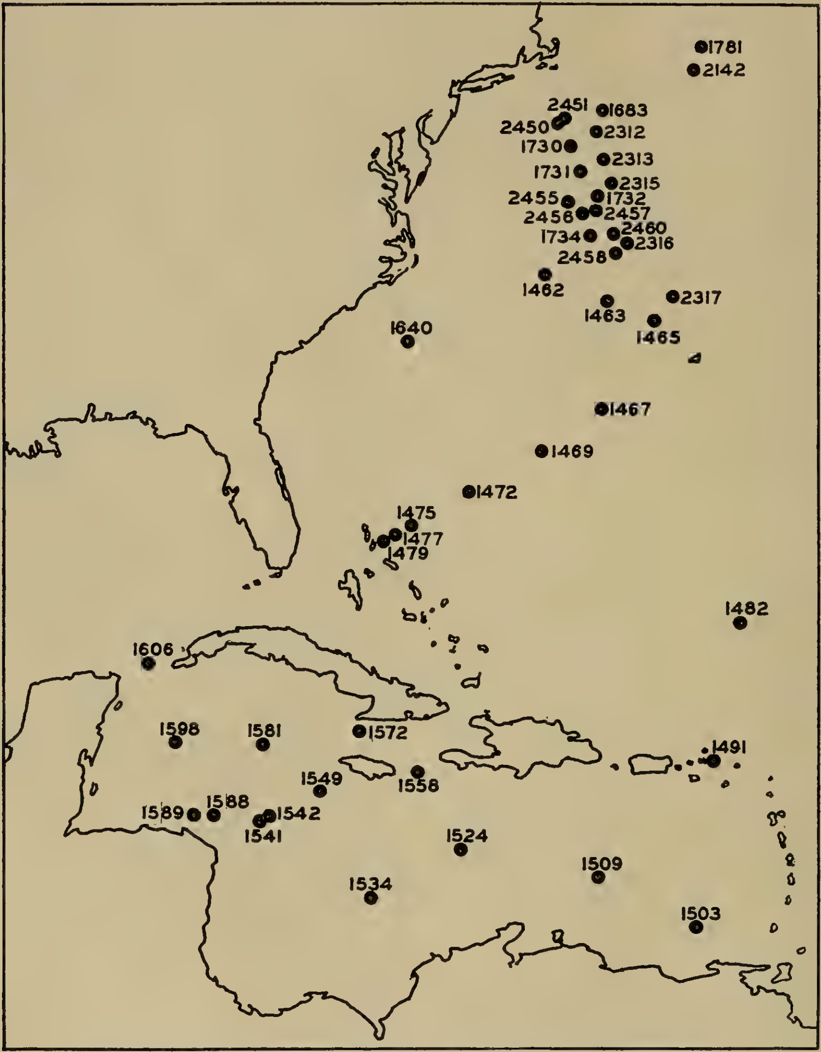
Station:	1712	1765	1888	1916	2037	2089	2182	2286
Depth in meters	7-8 1933	9-7 1933	12-7 1933	1-10 1934	3-24 1934	4-20 1934	5-25 1934	9-21 1934
0	0	0	.03	.02	.065	.09	.12	.19
10	0	.01	.02	.01	.07	.10	.12	.35
20	.165	.16	.03	.01			.13	
30	.22	.215	.05	.01	.065	.10	.11	.33
40	.21	.16	.035				.11	
50	.18	.04	.05	0	.06	.12	.06	.31
60	.17	.035	.035				.06	
80	.17	.035	.03	0	.06	.13	.09	.30
100	.065	.035	.02	0	.065	.13	.10	.06
125	.02	0		.02	.06	.07	.085	0
150	.03	.01	.02	.02	.04		.08	0
175	.04	.02					.05	0
200			0					0

Location 8

Station:	1716	1764	1853	1886	1914	2035	2087	2180	2227	2288
Depth in meters	7-9 1933	9-7 1933	10-19 1933	12-6 1933	1-10 1934	3-24 1934	4-20 1934	5-25 1934	6-29 1934	9-21 1934
0	0	0	.32	.085	.06	.065	.04	.065	0	0
10	0	0		.08	.06	.05	.05	.01	0	0
20	0	0	.28	.085			.11	.03	.24	
30	.115	.07		.07	.05	.06	.115	.11	.085	.10
40	.21	.01	.40	.08				.08	.07	
50	.215	0		.085	.03	.05	.115	.165	.05	.04
60	.12	.01	.15	.085				.17	.05	
80	.02	0	.16	.07	.01	.06	.08	.17	0	0
100	0	.02	.08	.085	.01	.01	.035	.15	0	0
125	0	.02				.01	.03	.135	0	.035
150	.04	.01	.01	0	0	0	0	.13	0	.01
175	.02	0			0	0	0	.09	0	0
200		0	.08	0		0	0	.05	0	.01
225					0	0	0	0		.04
250		0			0					0

of nitrite below this. Only during the winter months is nitrite found at the surface. The extremely sharp maximum zone at Station 1781 may be noted, at which nearly all the nitrite in the water column occurs in a twenty-meter layer, somewhat nearer the surface than at stations further to the south.

Stations in the Gulf of Maine, shown in Tables VI, VII and VIII are grouped into seven "locations." These include Location 22 on Georges Bank, which may be considered an adjunct to the Gulf of Maine. The separate stations in each of the locations are close enough together to be comparable, and were visited on different cruises at various times of the year, covering a total period of thirteen months or more. The data from six of these locations have been plotted in Figs. 5, 6, and 7 to show the seasonal variations of both nitrite and nitrate. The


1781
2142
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1683
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1503

Fig. 4. Location of stations from Cape Cod to the Caribbean.

data from Location 9 (not tabulated) are shown in Fig. 12, to indicate the relation between nitrite concentration, temperature and density.

These figures from the Gulf of Maine are but a small portion of the analyses made during a survey of that region, the results of which will appear at a later time.

TABLE VIII

Seasonal variation in the Gulf of Maine (continued). Nitrite concentration in microgram atoms per liter.

Location 15

Station:	1659	1781	1846	1899	1924	2052	2118	2137	2197	2296
Depth in meters	6-22 1933	9-10 1933	10-19 1933	12-7 1933	1-11 1934	3-26 1934	4-30 1934	5-6 1934	5-28 1934	9-23 1934
0	0	0	.19	.14	.11	.085	0	0	.01	0
10	0	0		.135			0	0	0	0
20	0	0	.14	.15	.065	.085	.07	0	0	
30	.01	0		.15			.11	0	0	0
40	.02	0	.19	.085	.06	.10			0	
50	.06	.18		.08			.115	.11	.065	.01
60	.09	.165	.11	.035	.06	.10			.115	.11
80	.05	.02	.01	0	.01	.10	.08	.16	.185	.265
100	.05	.02	.02	0	0	.07	.16		.085	.07
125	.01	.02				.01	.135	.04	.01	
150	.01	.02		0	0	0	.13	.05	.03	.035
175	.01	.01				0	.03	.03	.115	.02
200	.01	.02	.02	0	0	.07		.01	.115	.01
225						.06			0	
250	.01	0	.02	0	0	0		0		0

Location 22

Station:	1686	1742	1934	2069	2154	2214	2269
Depth in meters	7-27 1933	9-3 1933	1-13 1934	3-29 1934	5-10 1934	6-2 1934	9-18 1934
0	0	0	.03	—	.05	.05	.04
10	0	0		.035	.04	.06	.06
20	.03	.085	.035		.035	.04	
30	.035	.15		.03	.035	.05	.08
40	.04	.165	.03			.05	
50	.04	.18		.07	.03	.05	.15
60	.04	.185	.03	.035		.05	
80		.185				0	.21

The stations to the south of Bermuda, including those in the Caribbean (Table IX), present a rather different picture from that in the north. Generally, smaller amounts of nitrite are found, but frequently relatively large amounts at the surface. The maximum zone is near 100 meters, sometimes sharp, sometimes poorly defined.

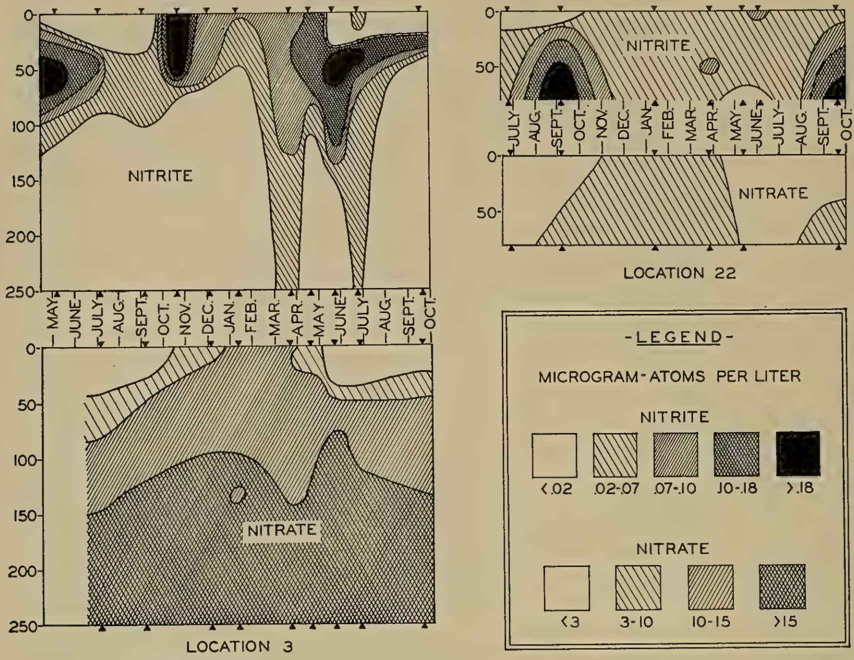


FIG. 5. Seasonal variation of nitrite and nitrate. Locations 3 and 22, Gulf of Maine. Ordinates, depths in meters.

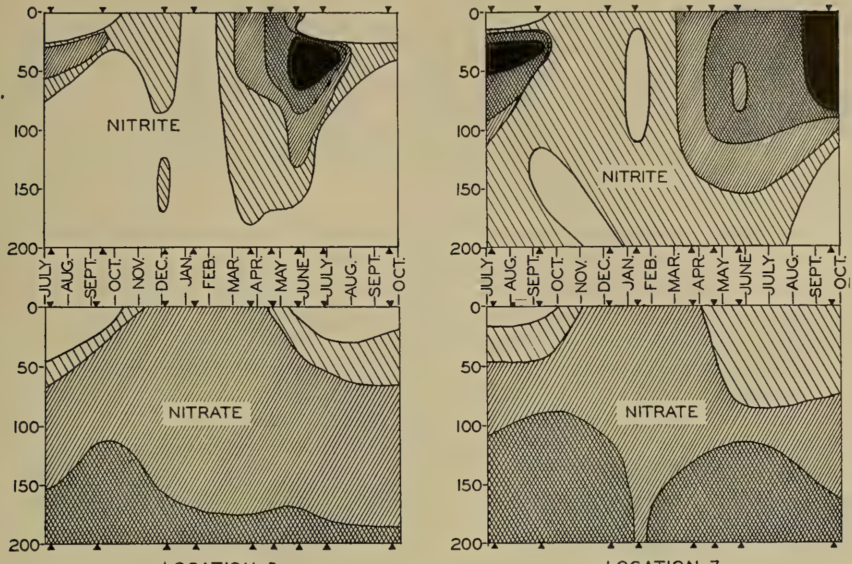
RELATION OF NITRITE TO OTHER FACTORS

Nitrate and Phosphate

Since nitrite participates in the metabolic cycle with the other " nutrient salts," it would seem important to compare its variations and behavior with that of the other nutrients, nitrate and phosphate.

Figures 9 and 10 show these relations at a number of stations in the Gulf of Maine at various times of the year. Certain conclusions are evident:

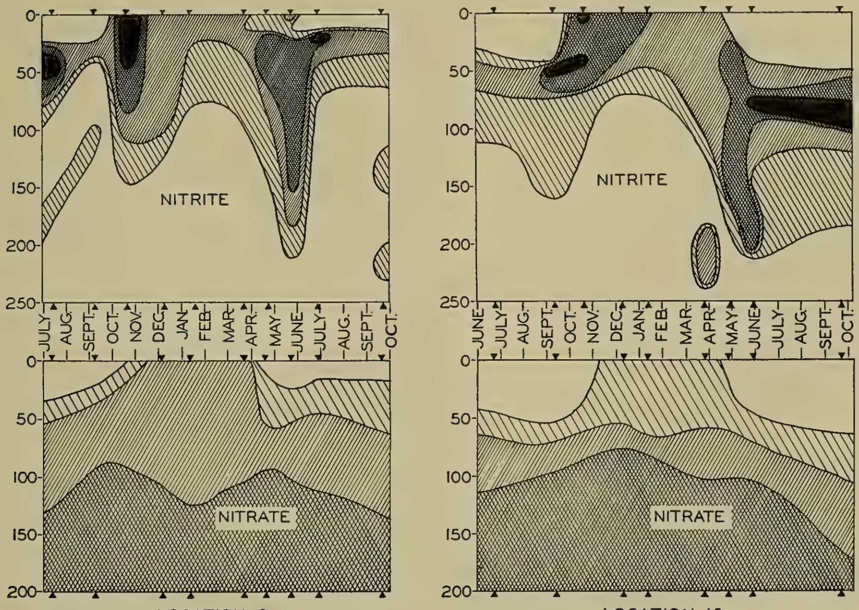
1. High nitrite concentrations in the upper water levels are associated with high concentrations of nitrate and phosphate. The converse is not always true, especially at certain times of the year. During the winter, when the surface is being replenished with nutrients by overturn of the water mass, nitrite is also in evidence, particularly as the season advances. In January, the production of nitrite is at a minimum, and since this corresponds to the time of greatest instability of the water mass it occasionally happens that little or no nitrite is found at any level,



LOCATION 5

LOCATION 7

FIG. 6. Seasonal variation of nitrite and nitrate. Locations 5 and 7, Gulf of Maine. (See Fig. 5 for legend.) Ordinates, depths in meters.



LOCATION 8

LOCATION 15

FIG. 7. Seasonal variation of nitrite and nitrate. Locations 8 and 15, Gulf of Maine. (See Fig. 5 for legend.) Ordinates, depths in meters.

even though there is abundant nitrate. This condition obtains at Stations 1908 and 1910.

2. High nitrate and phosphate in the *deeper* water is not associated with nitrite. The latter is seldom found in large amounts below 100 meters. It is evidently produced near the surface and works down through the unstable layer during the winter until stratification again sets in.

3. When diatom growth begins in the spring, with the utilization of nitrate and phosphate, nitrite also disappears in the surface water. At Station 2099 this process has apparently just begun, while at 2098 the season is further advanced, nitrate and phosphate are down almost to their summer values at the surface, and nitrite is absent. Figure 10 is compiled from a number of stations during the summer months to show the close correlation between the gradients of nitrite, nitrate and phosphate near the surface. The origin points for nitrite and nitrate coincide in these diagrams, but the phosphate curves are arbitrarily displaced to the left to facilitate comparison. The shape, slope and position of the curves indicate that the same conditions govern all three. As a consequence of this correlation, it is proposed that nitrite, which is normally produced near the surface, is utilized by the phytoplankton during the period of its growth, in the same manner as nitrate and phosphate, so that the upper levels become depleted. If the upper right-hand corner of the nitrite area shown in Fig. 9 for Station 1896 (or 1884) were cut off, and the curves for nitrate and phosphate deflected towards the origin, a condition would be represented exactly similar to those in Fig. 10. This is what would be expected during July or September. Abundant evidence of the utilization of nitrite by diatoms is

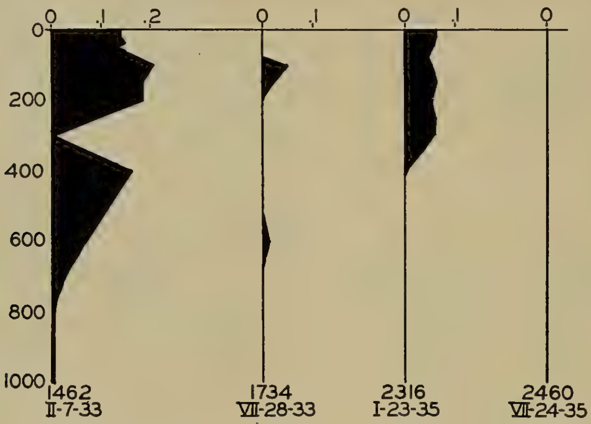


FIG. 8. Seasonal variation of nitrite at an oceanic station. (Group 3, Table V.) Ordinates, depths in meters.

TABLE IX

Stations south of Bermuda, including the Caribbean. Nitrite concentrations in microgram atoms per liter.

Station:	1467	1469	1472	1475	1477	1478	1482	1491	1503	1509	1524	1534	1541	1542	1549	1558	1572	1581	1588	1589	1598	1606
Depth in meters	2-14 1933	2-15 1933	2-17 1933	2-19 1933	2-19 1933	2-20 1933	3-3 1933	3-8 1933	3-15 1933	3-23 1933	3-29 1933	4-2 1933	4-4 1933	4-4 1933	4-5 1933	4-15 1933	4-25 1933	4-27 1933	4-29 1933	4-30 1933	5-1 1933	5-4 1933
0	0	.06	0	0	0	.01	.05	.07	.24	.35	.06	0	0	.04	.265	.04	.10	.165	0	.04	.01	.135
25	0	0	0	0	0	0	.01	.03	.14	.02	0	.02	0	.01		.02		0	0	0	0	.01
40	0	0	0	0	0	.02																
50					0		.05	.01	.18	.01	0	.01		0	.01	.01	.05	0	0	0	0	.01
100	0	.07	0	0	0	.035	0	.01	.05	0	0	.06		.32	.03	.02	.035	.185		.47	0	.11
150	0	.03	0	0	.11	.04	.03	.02	.035	.06	.035	.03		.02	.035	.04	.16	.035		0	.02	.035
200		.03			.01	.035	.035	.01	.01	.02	.02	.03		.02	.02	.02	.035	0		0	.01	.03
300	0	0	0	0	0	.01	.035	.09	.01	.01	0	.01			.02	.03	.03	0		0	0	.02
400	0	0	0	0	0	.01	.02	.02	.02	.01	0	.03			.01	.02	.02	0		0	0	.01
500		.01	0	0	0	.01	.02	.01	0	.01	0	.01			.02	.03	.01	0		0	.01	.01
600	.01	0	0	0	0		0	.04	.01	0	0	.02			.01	.02	.02	0		0	0	.01
700							.02	.04	.02											0		
800	0	0	0	0	.01		.01			0	0	.01			.01	.02	0				0	.01
900		0			.01		.01	.035		0												
1000	0	0	0	0	0		0	.01			0	.01			0	.02	.01					
1100							.03	.01														
1200	0	0	0	0	.01			.02			0											
1400	0	0	0	0	0		0	.02			0						.01					
1600	0	0	0	.01	.01		0	.02														
1800	0	0						.04														
2000	0	0	0	0	0		0				0						0					
2500			0	0	0		0				0											
3000	0	0		0	0		0				.01						.01					
3500											0											
4000	0	0									.01						.01					

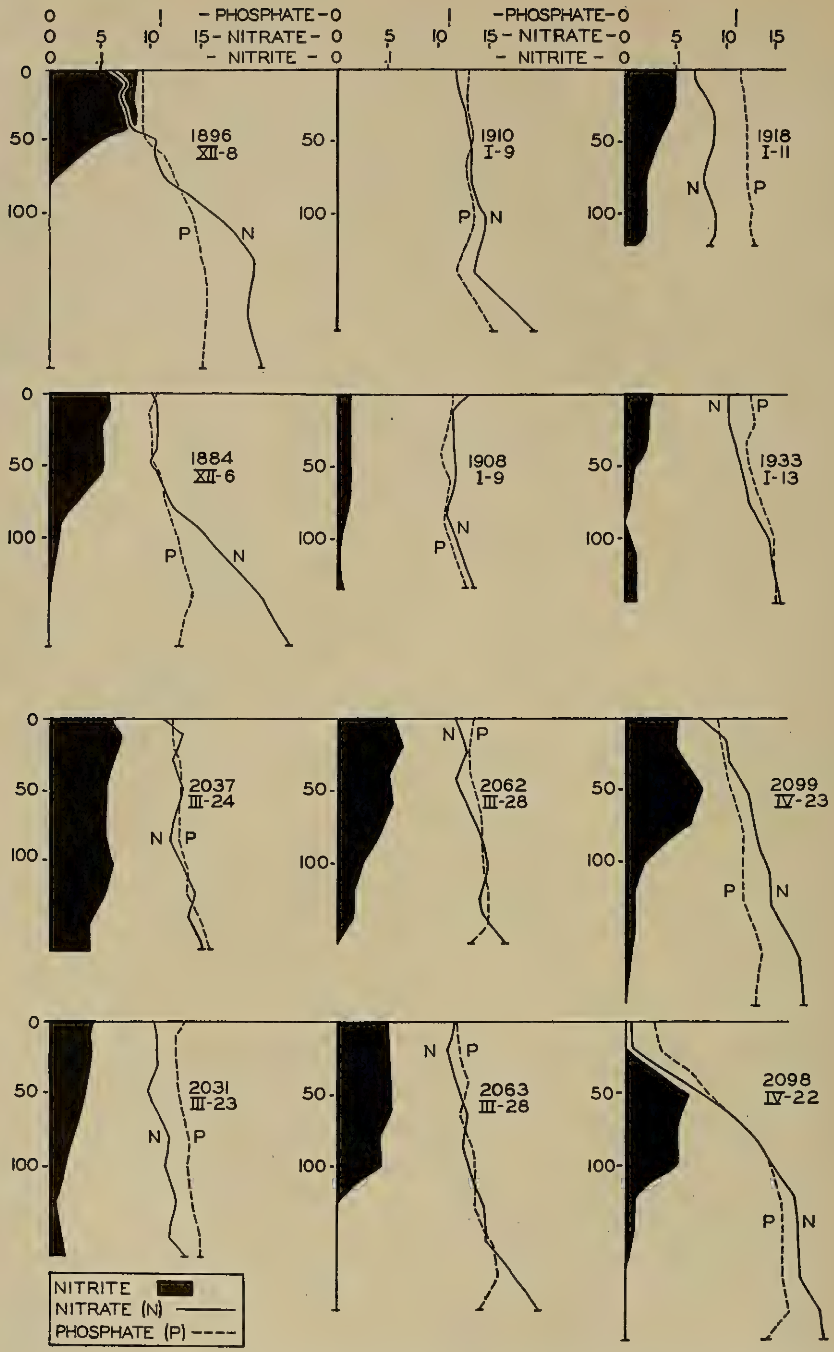


FIG. 9. Relation of nitrite to nitrate and phosphate. Various stations in the Gulf of Maine at different times of year. Ordinates, depths in meters. Positions of stations as follows:

Station	Position	Station	Position	Station	Position
1896	42°48′N 67°15′W	1910	42°43′N 69°15′W	1918	43°39′N 66°39′W
1884	43°00′N 68°37′W	1908	42°10′N 69°53′W	1933	41°25′N 69°02′W
2037	43°58′N 68°03′W	2062	42°44′N 68°44′W	2099	42°22′N 68°32′W
2031	43°18′N 69°23′W	2063	42°31′N 68°31′W	2098	42°50′N 67°19′W

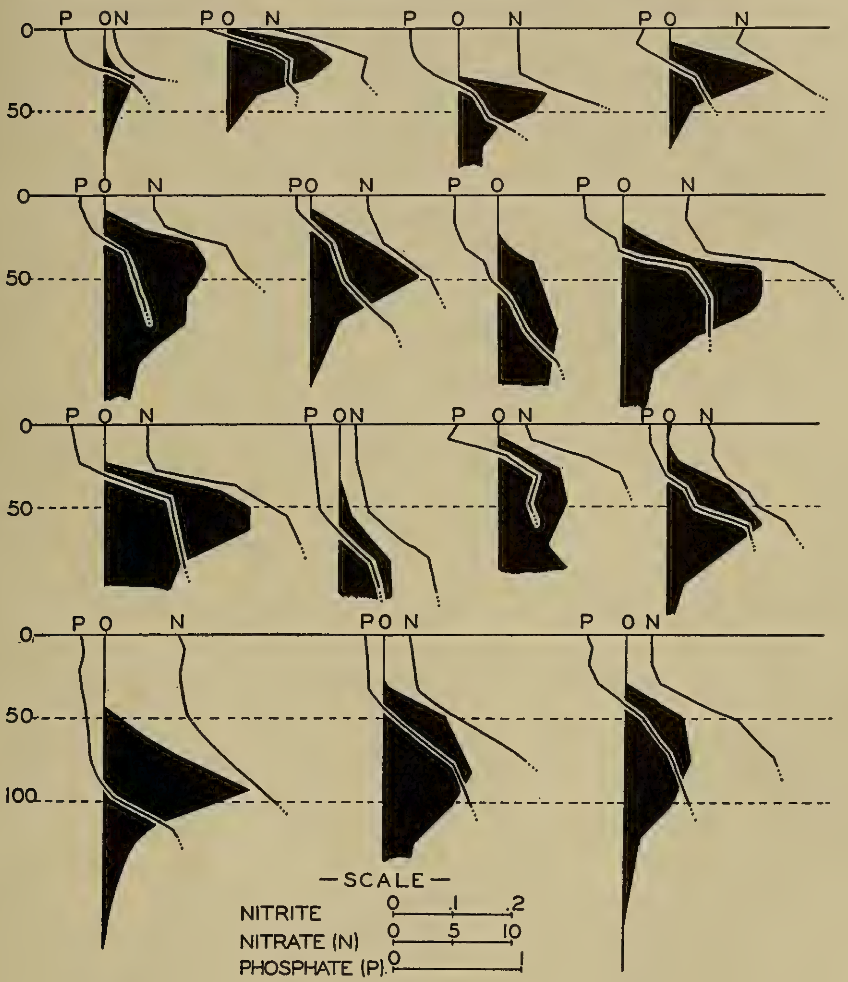


FIG. 10. Relation of nitrite to nitrate and phosphate. Various stations in the Gulf of Maine in the summer. (The origins for nitrite and nitrate coincide, but the phosphate curves are arbitrarily displaced to the left, to facilitate comparison of all three.) Ordinates, depths in meters.

afforded by Schreiber (1927), Cooper (1933), ZoBell (1935) and others.

But the Gulf of Maine is a rather specialized area; let us turn to the open ocean. Similar relations between nitrite, nitrate and phosphate are shown in Fig. 11 for a number of deep sea stations from Latitude 12 to 42 N. Most of these show a clearly-defined nitrite maximum, at about 100 meters, but all show nitrite at the surface, except the three northern stations, which were visited during the summer. At most of them there is a small but distinct amount of nitrite below 150–200 meters. Two stations, 1482 and 1491, are somewhat exceptional. At these, nitrite concentrations are rather low, but extend further down, with no well-defined maximum. The fertility of a region is obviously concerned in nitrite production and these stations are on the edge of the barren Sargasso Sea. Other stations in the Sargasso, as shown in Table IX, are largely devoid of nitrite.

Nearly all the nitrite formation takes place well above the level of maximum nitrate and phosphate concentration, which is found at about 800 meters south of the Gulf Stream and 400 meters north of it.

Density and Temperature

Figure 12 shows the relation between nitrite, density and temperature at stations in Location 9 at various times of year. It can readily be seen that:

1. As the inflection point in the density curve moves further down nitrite moves down also. This is another clear evidence that nitrite is formed near the surface and diffuses or mixes downward, more completely the more unstable the water column.

2. The production of nitrite goes on even at low winter temperatures, but with the rise in temperature nitrite is utilized as diatoms begin to grow.

3. The occurrence of a sharp nitrite maximum coincides with a high density gradient. If the depletion of nitrite above this is the result of diatom activity, this is localized and intensified by strong stratification.

Ammonia

Unfortunately, it was not possible to include simultaneous determinations of ammonia with those of nitrite and nitrate throughout this study. A few such determinations were made, however, using the method described by Krogh (1934). The results are shown in Table X, correlated with nitrite and nitrate. The low values for all three at the surface of Station 1739, and their increase toward the bottom, lend support to the view that they are all utilized at the surface and that ammonia is oxidized to nitrite and nitrate below. The increase of ammonia at the surface of Station 1742 (in Location 22) in September would seem to indicate that organic decomposition is going on more rapidly at this time, and ammonia being liberated, but that the second stage—appearance of nitrite—has not yet been reached. Or it may be that late in the summer, although nitrite is still being utilized, the ammonia is not drawn upon as a nutrient source. This was Cooper's assumption on finding the same condition in the English Channel.

Table X

Relation of nitrite to ammonia and nitrate. Concentrations in microgram atoms per liter.

Station	Depth in meters	Nitrite	Ammonia	Nitrate
1734 7/28/33	77	.01	.4	1.1
	340	0	.3	4.6
	855	0	.3	19.5
	2650	(0)	.6	20
	3280	(0)	.4	
	3725	(0)	.3	20.5
	4180	(0)	.6	23
1739 7/31/33	0	0	1.2	1.1
	10	0		1.1
	20	.02	1.4	2.5
	30	.44		4.0
	40	.39	4.3	5.7
	50	.36		5.7
	60	.24	4.1	7.1
	75	.36	4.3	6.4
1742 9/3/33	0	0	—	1.1
	10	0	2.8	0
	20	.085	3.0	2.1
	30	.15	3.2	4.3
	40	.165	3.2	6.1
	50	.18	3.8	7.5
	60	.185	4.2	7.1
	75	.185	—	7.5
2312	15	.04	.8	>13
	200	.05	0	10
2313	15	.02	.8	6.4
2314	15	.05	.7	2.8
2315	5	.08	.7	1.4
2316	5	.065	0	1.8
2317	5	.065	.3	1.7

At the various depths at Station 1734 the ammonia and nitrite are practically constant throughout, but the nitrite varies widely. From Station 2312 to 2317 the nitrate progressively diminishes, but both nitrite and ammonia are approximately constant. In fact, throughout all the data shown the nitrite is more closely related to ammonia than to nitrate.

Unpublished data in the hands of Dr. A. C. Redfield, on the distribution of ammonia in the Gulf of Maine in May and June, show a distinct tendency for ammonia to concentrate between 20 and 50 meters in a maximum zone similar to that for nitrite.

Nitrite Changes in Stored Samples

A number of previous investigators have tried to determine what happens to the nitrite in samples on standing. The results are altogether irregular and confusing. Buch (1923) reported the formation of as much as 28 mg./cu.m. of nitrite-N in two days, in samples standing in the dark. Harvey noted the appearance of nitrite in a few cases. Orr found increases in nitrite in diatom cultures, even after the diatoms had stopped growing. Braarud and Klem cite some experiments on nitrate reduction, but are not specific as to the relation of nitrite. Cooper followed 44 samples for 10–13 days, to some of which nitrite and ammonia had been added. He reports: " In all cases the changes in nitrite were small, little more than the experimental error." Keys, Christensen and Krogh (1935) followed the ammonia (and in a few cases nitrite, nitrate and oxygen) in samples stored for several days. Results were so variable that they finally conclude: " It appears to us that the only conclusion that can be drawn with regard to nitrogen metabolism in stored sea water is that there are usually a number of forces at work, some operating to reduce the free ammonia, others tending to release ammonia; the observed ammonia will always be a resultant of these forces. . . ."

Although with little hope of clearing up this confusion, a number of similar determinations on stored samples were made. The first of these were simply repeated analyses of the ordinary routine station samples made after a number of days standing. No particular precautions were taken to keep the samples in the dark, but they were exposed to probably less than normal illumination. Nearly 300 samples were followed in this way, with the results shown in Table XI.

One section of the table concerns samples which were originally " nitrite-free," or in which the initial concentration was not greater than .01. These are separated into three periods of standing, approximately 1, 2 and 3 weeks. Those which showed a significant increase in nitrite in that time (greater than .035 microgram atom per liter) amounted to 11 per cent, 12 per cent and 33 per cent of the totals. These 162 samples include many from the surface, with low nitrate, and many from deep levels, with high nitrate. There was no difference in the behavior of these two classes, a fact which has a bearing on the question of reduction of nitrate to nitrite.

The other group of 178 samples contained initial concentrations of nitrite from .01 to .91. Changes took place in both directions. But relatively fewer cases of increase, and more of decrease, were found as the time of standing lengthened. The predominating tendency under these conditions is for nitrite, if initially present, to disappear on standing. If initially absent, it may appear in small amounts.

TABLE XI

Nitrate changes in stored samples

A. Samples with initial concentration = 0 (<.01 microgram atoms per liter)

	5–7 days	12–15 days	20–24 days
Total number	109	41	12
No. increased >.035 microgram atom per liter	12	5	4
Percentage of total	11%	12%	33%

B. Samples with initial concentration >0 (.01–.91 microgram atom per liter)

	5–7 days	12–15 days	20–24 days
Total number	119	25	34
No change (Increase or decrease <10 per cent of initial concentration)	49	9	9
Number increased, over initial concentration, 10–50 per cent	18	1	1
Number increased, over initial concentration, >50 per cent	11	1	0
Number decreased, of initial concentration, 10–50 per cent	31	14	17
Number decreased, of initial concentration, >50 per cent	10	0	7
Percentage of total number which *increased* more than 10 per cent of initial concentration	24%	8%	3%
Percentage of total number which *decreased* more than 10 per cent of initial concentration	34%	56%	70%

The results of these experiments do not lead to a conclusion much more definite than that of Keys, Christensen and Krogh.

Subsequently, however, several series of more carefully controlled experiments were carried out. Samples of filtered surface water were placed in Pyrex flasks. Some of these contained added amounts of ammonia-nitrogen, others added nitrate. Half the samples were sterilized in an autoclave, and of the total number, half were stored in the dark and half were placed in the sunlight as much as possible during the period of storage. Analyses for nitrite were made at intervals up to 19 days. Two series of experiments of this sort were carried out, with the following results:

1. The samples stored in the dark, whether sterile or not, showed no significant change in nitrite, even in the presence of added nitrate or ammonia.

2. Those kept in the light invariably showed a change in nitrite. If it was high at the beginning it decreased, but not so sharply in the presence of excess nitrate. If the nitrite concentration was low at the start it increased slightly, but *more* so in the presence of added nitrate. In fact, after 19 days the nitrite reached the same value, whether by increase or decrease. This seems to depend, however, upon the presence of sufficient nitrate, and is independent of the concentration of ammonia. The conditions surrounding the attainment of this apparent "steady state" will be the subject of further investigation.

3. The rate of disappearance of nitrite was unaffected by the presence of excess ammonia, and only a very slight increase was found in samples of low original nitrite content. In view of the conditions of these experiments this result is not necessarily in conflict with those of ZoBell (1933), who reported the photochemical oxidation of ammonia to nitrite under certain conditions.

4. An effort to follow the changes in nitrate led to inconclusive results. In one series of experiments the sum of nitrite and nitrate (determined by Harvey's "nitrate" method, using reduced strychnine as a color reagent) remained constant, although the concentration of nitrite alone decreased considerably; but in another series the increase in nitrate did not compensate for the disappearance of nitrite.

Thiele (1907) was apparently the first to show that nitrate can be reduced to nitrite by ultraviolet light. Moore (1918) found that sunlight can accomplish the same result, but that the shorter wave-lengths are the most active. More recently, Villars (1927) has studied the photochemical decomposition of KNO_3 in considerable detail and has shown that wave-lengths less than 3,000 Ångstroms are effective in the reduction. About 1 per cent of the total energy of the solar spectrum at sea level falls within this range.

Nitrite in the Sea Bottom

It is evident that at shallow stations, at least, a great part of the decomposition of organic matter takes place on the bottom. A study of the nitrite content of bottom sediments might therefore be significant

Brandt gives figures for the ammonia and albuminoid-nitrogen content of " bottom water " (that in contact with the bottom surface) and of " interstitial water " (that contained in the bottom cores brought up) showing from three to five times as much in the latter as in the former. This relation was apparently independent of depth, for the bottom samples varied from 45 to 3,000 meters. He also cites determinations on muds from the North Sea showing 2–4 mg. of ammonia-N and 5–10 mg. of nitrate-N per kilogram of moist mud.

A number of bottom samples were obtained from various sources and determinations made of the nitrite and nitrate in the interstitial and adjacent water. The results are contained in Table XII.

Figures are given for the nitrite and nitrate not only in the bottom samples, but also in the lowest water bottle, which will vary from 2 to 20 meters from the bottom.

Care must be taken in comparing the results, for different methods were used in obtaining and treating the samples. In some cases, the interstitial water was removed from the whole mud core by suction and the nitrite concentration expressed in terms of the volume of water thus recovered. In other cases, samples of mud and the water in contact with it were collected, either in the coring device, the Pettersson grab, or a special mechanism which sucks up a sample of mud and water off the bottom. Nitrite was determined in this water, after filtration.

Although the data from deep stations are fragmentary they are sufficient to show that conditions in deep bottoms are different from those in shallow ones. The single determination at Station 1731 is not quantitative, but it at least shows that there is very little nitrite in the mud, compared with Stations 1331, 2220 and 2227, where similar technic was used. The condition of the sample from Station 1734 is doubtful, but it should approximate those from A-8 and A-25. Nevertheless, the content of nitrite is very low, and the nitrate is very little greater than that in the lowest water-bottle.

The results from Stations 2220, 2224 and 2227 are interesting in showing the enormous quantities of nitrite in the interstitial water of shallow bottoms.

A comparison of these quantities with those in the supernatant " bottom water " shows that the rate of nitrite formation in the mud is very much higher than its rate of diffusion into the surrounding water.

At Station A-8 and again at A-25 two samples were obtained as close together as possible. The variation in their nitrite and nitrate content is partly due to the method of sampling, which affords a mixed sample of mud and water sucked off the bottom. Nitrite was determined in such a sample after filtration, and although this contains " in-

TABLE XII

Nitrite and nitrate in the sea bottom. Concentration in microgram atoms per liter.

Station	Depth in meters	In lowest water-bottle		In bottom		Method of obtaining sample for analysis
		Nitrate	Nitrite	Nitrate	Nitrite	
1734	4630	20.5[2]	0?	23.0[1]	.05	Water-bottle closed on bottom; brought up mud and water.
1731	3840				.04	Portion of core extracted with surface water.
1336	65	5.0	.28	25.0	1.8	Water brought up with sand in Pettersson grab.
1331	200		.115		.63	Portion of core extracted with surface water.
2220	218		.04		.065	Supernatant "bottom water" on top of core.
2220	218		.04		28.3[3]	Interstitial water removed from core by suction.
2224	195		0		.035	Supernatant "bottom water" on top of core.
2224	195		0		3.3	Interstitial water removed from core by suction.
2227	210		.01		.035	Supernatant "bottom water" on top of core.
2227	210		.01		10.8[4]	Interstitial water removed from core by suction.
A-8*	32	A B	.235 .235	4.6 10.3	.26 .74	The sampling device sucks up a sample of mud from the surface of the bottom, along with water in and around it. Contains interstitial and bottom water in varying proportions.
A-25*	45	A 10.3 B 10.3	.13 .13	17.2 24.6	.20 .25	
Near K-3†	15	A B C			.065 .12 .285	
Near K-3*	15	A B			.75 .77	

* Two samples, A and B, taken as close together as possible.

† Three samples, A, B, C, taken as close together as possible.

[1] Ammonia-nitrogen concentration, 0.65.

[2] Ammonia-nitrogen concentration, 0.35.

[3] Total extractable nitrite-N, 1.5 mgm. per kgm. of dry mud.

[4] Total extractable nitrite-N, .25 mgm. per kgm. of dry mud.

terstitial" and "bottom" water in varying proportions the irregularity of results would nevertheless seem to indicate great local differences in bottom conditions.

Similarly, near Station K-3, three samples were taken simultaneously at one place and two at another. The first three were quite different in nitrite content, while the latter two were practically identical.

The general conclusion to be drawn is that the decomposition of organic matter on the sea bottom involves oxidation of nitrogen compounds to nitrite and nitrate, and that this process is much more intense on shallow bottoms than deep. Nearly all of the nitrification must have taken place before organic matter reached the bottom in the latter cases.

The samples from near Station K-3 were analyzed again after having stood for one day. The nitrite in three had approximately doubled; in the fourth it had increased 50 per cent. The water from the fifth mud sample had been separated from the mud at the time of the first analysis, and the nitrite in this water had *decreased* 50 per cent after one day. Evidently nitrite is formed rapidly and continuously in the mud, diffusing into the water in contact with it. In the water, however, the nitrite is rapidly destroyed, presumably by oxidation to nitrate.

Samples of white coral mud from Castle Harbor, Bermuda, at a depth of about 5 meters, were extracted by distilled water and surface sea water, respectively. The distilled water extract contained: nitrate, .85; nitrite, 0. The surface water extract contained: nitrate, 15; nitrite, .085. The surface water itself was nitrite-free and contained .7 microgram atom per liter of nitrate-N. The more efficient extraction by sea water shows the same phenomenon of "base-exchange" so common in the extraction of soils.

Nitrite at the Surface

The question of the occurrence of nitrite in the immediate surface layer seems to merit particular consideration.

The first intimation of irregularity occurred on the section from Cape Hatteras to Bermuda (see Fig. 4). At Stations 1462 and 1463 suspiciously high surface values were obtained. These were traced, apparently, to contamination, and were finally rejected. But later, at Station 1465, there could be no doubt of a fairly high concentration at the surface, with very little immediately below. However, no surface nitrite could be found in the vicinity of Bermuda or at the immediately following stations, except 1469. Between this station and 1472 surface samples were picked up every hour or two, while the ship was in motion, without finding more than a trace of nitrite. The same was true of a number of samples taken up between Stations 1473 and

1475. Much later, six samples taken in the same way between Stations 1730 and 1731 were nitrite-free.

Throughout the Caribbean cruise nitrite was found at the surface at the majority of the regular stations, but at four places where surface samples alone were taken none was found. In all, out of thirty surface samples taken with the ship in motion, detectable traces of nitrite were present in only six, and only once a concentration of more than .01. Whether the occurrence of considerable amounts of surface nitrite in the Caribbean is constant or limited to a certain time of the year (as in the north) cannot be said from the results of this one cruise, but the strange irregularity suggests that special factors—perhaps photochemical—may operate in the immediate surface, a matter which will later be investigated more fully. Highly irregular variations in sur-

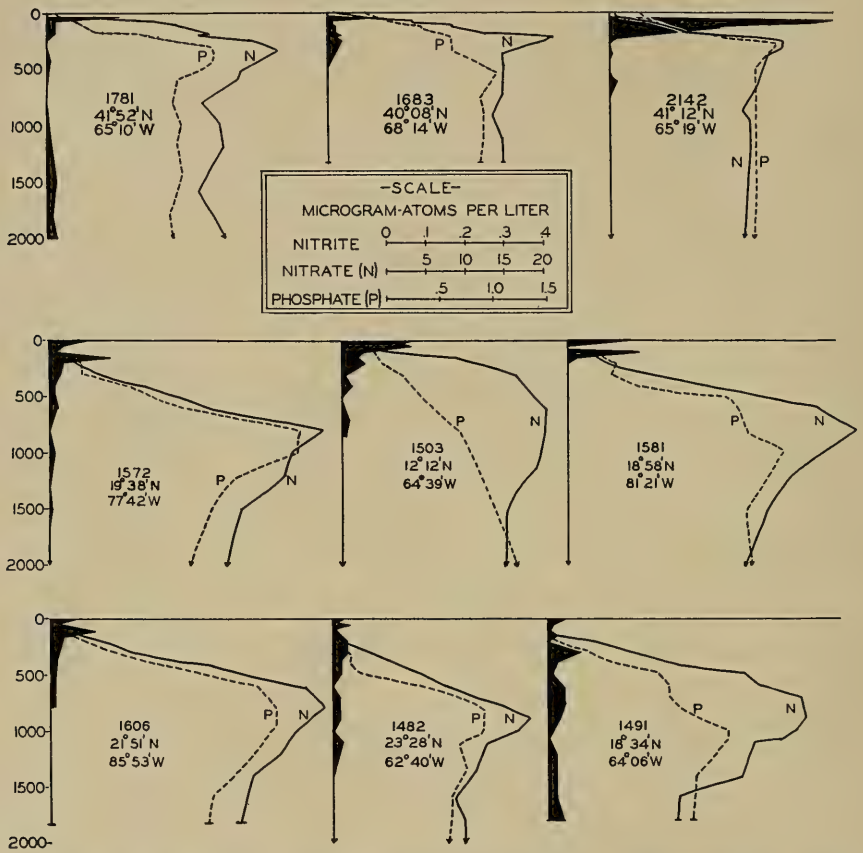


FIG. 11. Relation of nitrite to nitrate and phosphate, at deep stations. Ordinates, depths in meters.

face nitrite have also been reported by Matsudaira and Yasui between Nagasaki and Shanghai.

The vicinity of Bermuda is an area especially devoid of nitrite and other "nutrients." A number of surface samples were collected from the harbors and bays of the Bermuda Islands, and even from their fresh water streams and ponds, with no more than traces of nitrite, and minimal amounts of nitrate.

Seasonal Changes in Nitrite

Figures 5, 6, and 7 contain the data from Locations 3, 5, 7, 8, 15 and 22 in the Gulf of Maine, arranged to show seasonal variations of nitrite and nitrate. Figure 12 shows the progressive changes at Location 9 in a different way. The following points are revealed:

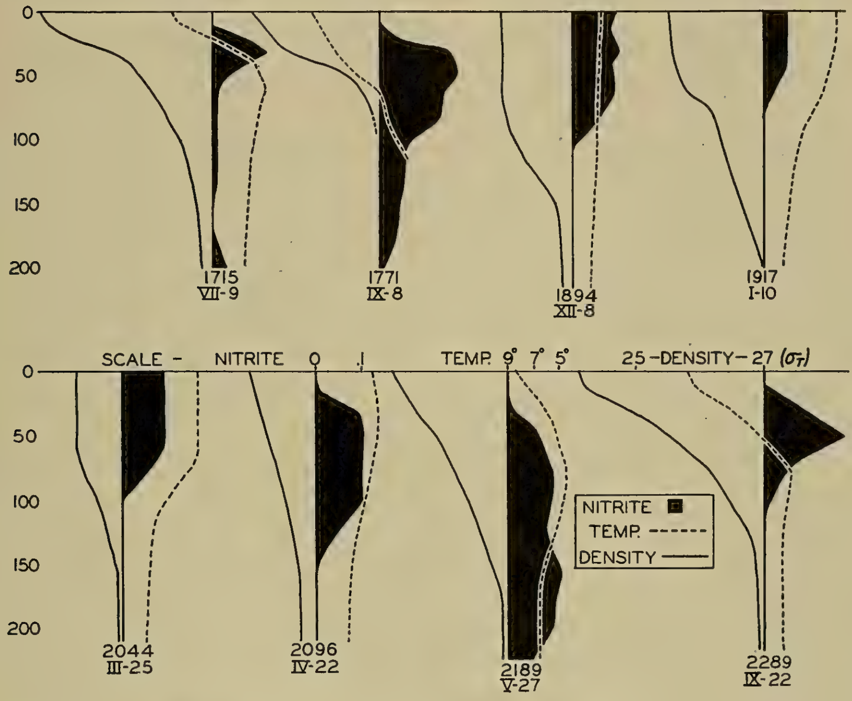


FIG. 12. Relation of nitrite to temperature and density (σ_T). Location 9, Gulf of Maine, at various times of year. Ordinates, depths in meters.

1. A minimum of nitrite occurs in January and February, concentrated at the surface. This corresponds to the period of maximum nitrate and phosphate, when the water mass is nearly homogeneous. Nitrification is going on at the surface, but only very slowly.

2. There follows a slow increase in nitrite until April, when more rapid production accompanies the diatom outburst and continues during the whole period of growth.

3. Simultaneously with the growth of diatoms, however, the removal of nitrate from the surface begins, and nitrite also starts to disappear in the same regions. At Locations 3 and 5 the removal of nitrite lags somewhat behind the nitrate, possibly because the latter is utilized more readily. At Location 7, during July, August and September of the second year, considerable nitrite is still present at the surface, but since the nitrate has not yet been completely used up it would seem that the nitrite store has not been heavily drawn upon.

4. The maximum of nitrite occurs between June and September, varying with local conditions at different stations. There is evidence that the seasons are somewhat displaced in different parts of the Gulf of Maine and that the growth of organisms does not take place uniformly throughout.

5. The utilization of nitrite in the surface layers, simultaneously with its production throughout a much deeper water mass, results in the appearance of a maximum zone, generally between 30 and 50 meters.

6. The occurrence of considerable nitrite in the surface layers at Locations 3, 8 and 15 late in October is noteworthy. This did not occur in all parts of the Gulf but does not seem to be fortuitous. It may well be associated with the death and disintegration of organisms after the close of the growth period. Soot-Ryen reported the same thing in his study of one of the Norwegian fjords. It is possible that this phenomenon was missed at Locations 5 and 7 from lack of data.

7. Location 22, being shallow, shows only the upper part of the picture. Maximum nitrite production has taken place here a little later.

The results of Soot-Ryen are altogether similar to these, with a slight displacement of the seasons. He found a minimum of nitrite in January, increasing through the spring and early summer to a maximum in August, but with depletion of the surface during the summer. This was followed by another short period of nitrite production in the upper layers in November.

Verjbinskaya's data on seasonal variation of nitrite in the Barents Sea are somewhat incomplete, but as far as they go they indicate the same general sequence: nitrite-free water in December and January, building up to a maximum in August, but with surface depletion during the summer.

Cooper's very complete study in the English Channel deals with a station too shallow to show vertical distribution of nitrite, but in all other respects is in complete accord with the above findings. It shows the same simultaneous removal of nitrite and nitrate from the surface in the summer, followed by rapid production of nitrite and ammonia in October, and a minimum during December and January.

No study of seasonal variations at a truly oceanic station seems ever to have been made. Figure 8 shows the nitrite concentration in the upper 1,000 meters of the four corresponding stations in Group 3 (Table V). The only feature in common with the data from shallower stations is the surface depletion during the summer. Nitrite production evidently takes place in the upper levels during the winter. The hydrographic and biological conditions are so different here that a much more complete investigation is necessary.

The Position of Nitrite in the Nitrogen Cycle in the Sea

Brandt, in his monumental work on nitrogen metabolism in the sea, summarized all the earlier results and finally came to the conclusion that ammonium salts are oxidized to nitrite and nitrate by nitrifying bacteria in the sea bottom. Impressed by the fact that such organisms had never been found in the water itself, but that nitrite was frequently present at the surface, he proposed that bacteria might oxidize dissolved organic matter directly to nitrite and nitrate without ammonia as a necessary intermediate.

One view of the significance of nitrite was that expressed by Atkins: that its presence in sea water indicates the production of nitrate. Its absence from deep oceanic samples, on the other hand, shows that nitrification is complete. This assumes, of course, that nitrite is solely produced by oxidation of ammonia, or perhaps from dissolved organic matter directly.

Others, such as Brujewicz (1931) and Braarud and Klem (1931), have rejected this view and incline to that which explains nitrite formation as a result of the reduction of nitrate. Either theory will explain many of the results. Organic matter in the zone of nitrite production, present in considerable amounts, may either be the direct source of nitrite, with ammonia as a likely intermediate, or it may serve as the necessary source of energy for the nitrate-reducing bacteria. These have, in fact, been found there in small numbers.

The presence or absence of the necessary organisms is a bacteriological problem which this present paper cannot go into, but there are a number of considerations from the observed chemical data which favor oxidation as a source of nitrite, rather than reduction.

First, it is unlikely that the upper layers of the water, where oxygen tension is high, would be a suitable environment for reduction processes to take place. The bacterial reduction of nitrate to nitrite is known to be possible under nearly anærobic conditions, and in the presence of enormous quantities of organic matter, but it is not certain that the same thing would take place in surface sea water.

The relation of nitrite and nitrate in bottom muds to each other and to the surrounding water, mentioned above, makes it clear that both are being *formed* in the bottom, obviously by oxidation of organic matter. Since nitrite is an intermediate in this process, it is scarcely possible that the large amounts found here could have been produced by a *subsequent* reduction of the nitrate; and if the nitrite in shallow muds is the result of oxidation it is very likely that all in the water above is of the same origin, for there is no evidence of any discontinuity of chemical mechanism from top to bottom. A comparison of the profiles of stations of varying depths suggests that the concentration of nitrite depends on a balance between the rate of chemical transformation and the rate of descent of the matter being transformed.

The relation between ammonia and nitrite which Cooper's results bring out, as well as the data presented above, especially the increase of both immediately after the summer growth period, are best explained on the assumption of oxidation of the former. The correspondence of the intermediate maximum zone of both nitrite and ammonia, already referred to, supports this view.

The general correlation of low concentrations of both ammonia and nitrite in the deep sea, and of higher concentrations of both, frequently, in other regions, is of some significance.

Furthermore, dissolved organic matter is nearly as abundant at great depths as near the surface, and nitrate is present in large quantity. This would seem to be an optimum condition for reduction of nitrate; but nitrite is seldom found here. It may, of course, be true that dissolved forms of organic matter are unsuitable for nitrate-reducing bacteria.

Finally, the following theory is presented to explain the observed facts:

During the winter, when biological activity is at a minimum, the decomposition of organic matter is going on very slowly. This takes place largely near the surface, either as the result of oxidation of excretory products liberated here, or the death of organisms, with disintegration and decay beginning before they have sunk far. Quantitatively, this same process goes on with greater rapidity as the season progresses. Nitrite is an early stage in the oxidation of this organic matter, presumably after the liberation of ammonia as a previous step. This is in keeping with Wattenberg's (1930) conclusion that ammonia formation is mainly a surface phenomenon. Later in the winter and early spring, as the unstable column becomes deeper, these processes take place through a deeper vertical range.

Still later, when diatoms begin to grow actively they utilize the nitrite in the surface water, along with nitrate. Occasionally, when there are plenty of other nutrients, nitrite is not exhausted. Such is the case in the Antarctic, where determinations made on the "Discovery" show high concentrations of nitrite above 100 meters during the summer, but associated with very large quantities of nitrate and phosphate.

Organic matter works its way downward, by settling, if it is particulate, or by diffusion or mixing, if dissolved. The appearance of nitrite by oxidation therefore extends below the zone of its utilization. At greater depths it disappears again by further oxidation to nitrate.

Nitrate concentration continues to increase to still greater depths, reaching a maximum at 800–1,000 meters, generally. Nitrification extends further than the occurrence of nitrite would indicate.

The decomposition of organic matter takes place in several steps, and we may suppose that the products of the first stages are more rapidly oxidizable than those of the later ones. This may be the result of either the chemical nature of the material or its concentration, which would be greater at the start of the process, before it has become diluted. The formation of nitrite in significant amount is associated with the initial stages of the decomposition of organic matter, probably because of their greater rapidity, but the whole process of nitrification does not complete itself until greater depths are reached, and by this time the nitrite, formed in the first part of the process, has been further oxidized to nitrate.

Vertical differences in temperature may also play a part in determining the rates of decomposition.

Recently, ZoBell (1935) has suggested that diatoms may reduce nitrate to nitrite extracellularly and then absorb it as such. Such a theory would explain many of the observed phenomena with respect to nitrite distribution, to be sure, particularly its close association with phytoplankton. Its acceptance, however, will depend upon its satisfactory explanation of the relation of nitrite to ammonia. Such data as we now have covering this point seem difficult to reconcile with this view, but until we have had an opportunity to extend them we prefer to withold judgment.

Acknowledgment

I should like to express my thanks to Doctors H. P. Smith and H. E. Mahncke and Mr. E. F. Beach, who carried out some of the

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LITERATURE CITED

Atkins, W. R. G., 1930. Seasonal changes in the nitrite content of sea water. *Jour. Mar. Biol. Ass.*, **16**: 515.

Böhnecke, G., E. Hentschel and H. Wattenberg, 1930. Über die hydrographischen, chemischen und biologischen Verhältnisse an der Meeresoberfläche zwischen Island und Grönland. *Ann. d. Hydrog. und Marit. Meterrol.*, **58**: 233.

Braarud, T., and A. Klem, 1931. Hydrographical and chemical investigations in the coastal waters off Möre and in the Romsdalsfjord. *Hvalrådets Skrifter*, No. 1, p. 48.

Brandt, K., 1916–20. Über den Stoffwechsel im Meere. *Wiss. Meeresuntersuch., Kiel*, **18**: 185.

Brandt, K., 1927. Stickstoffverbindungen im Meere. *Wiss. Meeresuntersuch., Kiel*, **20**: 201.

Brujewicz, S. W., 1931. Hydrographic Work of the State's Oceanographic Institute of U. S. S. R. in the Barents Sea in 1927–30. *Rep. First Session, State Ocean. Inst.*, No. 1.

Buch, K., 1923. Methodisches über die Bestimmung von Stickstoffverbindungen im Wasser. *Merentutkimuslaitoksen Julkaisu*, No. 18: 21.

Cooper, L. H. N., 1933. Chemical constituents of biological importance in the English Channel, November, 1930, to January, 1932. Pt. 1. *Jour. Mar. Biol. Ass.*, **18**: 677.

Discovery Committee, 1932. Station List. Discovery Reports, 5: No. 1.

Harvey, H. W., 1926. Nitrate in the sea. *Jour. Mar. Biol. Ass.*, **14**: 71.

Keys, A., E. H. Christensen and A. Krogh, 1935. The organic metabolism of sea water, with special reference to the ultimate food cycle in the sea. *Jour. Mar. Biol. Ass.*, **20**: 181.

Krogh, A., 1934. A method for the determination of ammonia in water and air. *Biol. Bull.*, **67**: 126.

Matsudaira, Y., and Z. Yasui, 1932–33. Surface observations between Nagasaki and Shanghai. *Jour. of Oceanog.* (Kobe), **4**: 473; **5**: 439.

Moore, B., 1918. The formation of nitrites from nitrates in aqueous solution by the action of sunlight, and the assimilation of the nitrites by green leaves in sunlight. *Proc. Roy. Soc., B*, **90**: 158.

Orr, A. P., 1926. The nitrite content of sea-water. *Jour. Mar. Biol. Ass.*, **14**: 55.

Orton, J. H., 1924. An account of investigations into the cause or causes of the unusual mortality among oysters in English oyster beds during 1920 and 1921. *Fisheries Investigations* (*Ministry of Agric. and Fisheries*), Ser. 2, 6: No. 3, 130.

Raben, E., 1914, 1918. Über quantitative Bestimmungen von Stickstoffverbindungen im Meerwasser und Boden, etc. *Wiss. Meeresuntersuch., Kiel*, **16**: 207; **18**: 1.

Rakestraw, N. W., 1932. Phosphorus and nitrogen in the neritic waters of the Gulf of Maine. *Int. Rev. Hydrobiol. und Hydrogr.*, **27**: 151.

Rakestraw, N. W., 1933. Studies on the biology and chemistry of the Gulf of Maine. I. *Biol. Bull.*, **64**: 149.

Robinson, R. J., and H. E. Wirth, 1934. Report on the free ammonia, albuminoid nitrogen and organic nitrogen in the waters of the Puget Sound area, during the summers of 1931 and 1932. *Jour. du Conseil*, **9**: 15.

Robinson, R. J., and H. E. Wirth, 1934. Free ammonia, albuminoid nitrogen and organic nitrogen in the waters of the Pacific Ocean off the coasts of Washington and Vancouver Island. *Jour. du Conseil*, **9**: 187.

Schreiber, E., 1927. Die Reinkultur von marinem Phytoplankton und deren Bedeutung für die Erforschung der Produktionsfähigkeit des Meerwassers. *Wiss. Meeresuntersuch., Helgoland,* N. F., **16**: No. 10.

Soot-Ryen, T., 1932. Changes in the nitrite content through the year in North-Norwegian Fjords. *Jour. du Conseil,* **7**: 246.

Thiele, H., 1907. Einige Reaktionen im ultravioletten Lichte. *Ber. deutsch. chem. Ges.,* **40**: 4914.

Verjbinskaya, N., 1932. Observations on the nitrite changes in the Barents Sea. *Jour. du Conseil,* **7**: 47.

Villars, D. S., 1927. The photolysis of potassium nitrate. *Jour. Amer. Chem. Soc.,* **49**: 326.

ZoBell, C. E., 1933. Photochemical nitrification in sea water. *Science,* **77**: 27.

ZoBell, C. E., 1935. The assimilation of ammonium nitrogen by Nitzschia closterium and other marine phytoplankton. *Proc. Nat. Acad. Sci.,* **21**: 517.

THE BIOLOGY OF OITHONA SIMILIS IN THE GULF OF MAINE AND BAY OF FUNDY

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Introduction

Although *Oithona similis,* one of the two microcalanids found breeding in the Gulf of Maine, is small in size, the relative abundance of larvæ indicates that at times it forms a significant factor in the natural economy of the region.[2] It is also particularly favorable for quantitative study because adults, larvæ, and egg cases all appear to be sampled representatively with fine nets (No. 10 silk) and the pump.

This paper forms the third of a series on the biology of zoöplankton species in the Gulf of Maine and Bay of Fundy with special reference to production and dispersal, and is based on material obtained between July 28, 1931 and September 29, 1932, in the course of investigations carried on for the International Passamaquoddy Fisheries Commission. For a description of the area covered, location of stations, and methods, the reader is referred to a previous report (Fish, 1936*a*).

Regional Distribution of the Adult Stock

Oithona similis is almost "world wide" in distribution (Bigelow, 1926), but on the American coast appears to be largely centered north of Cape Cod. South from this point it is gradually replaced, in neritic waters at least, by *O. brevicornis,* a species not yet reported north of the Cape. At Woods Hole in 1922 and 1923 (Fish, 1925, Fig. 47) the latter species occurred with southern forms during the summer from late June to mid-October, and *O. similis* with the boreal population in autumn and winter. Farther south in Chesapeake Bay *O. similis* is decidedly a winter form limited largely to the inner Bay and is exceeded by *O. brevicornis,* which occurs everywhere in that region throughout the year (Wilson, 1932).

North of Cape Cod McMurrich (unpublished data) found *O. similis* in small numbers in Passamaquoddy Bay regularly during the colder months from November until March, but very rarely during the spring and summer. Elsewhere in Canadian waters, however, it has been reported widespread offshore during the summer (Herdman, Thompson, and Scott, 1898; T. Scott, 1905; Pinhey, 1926).

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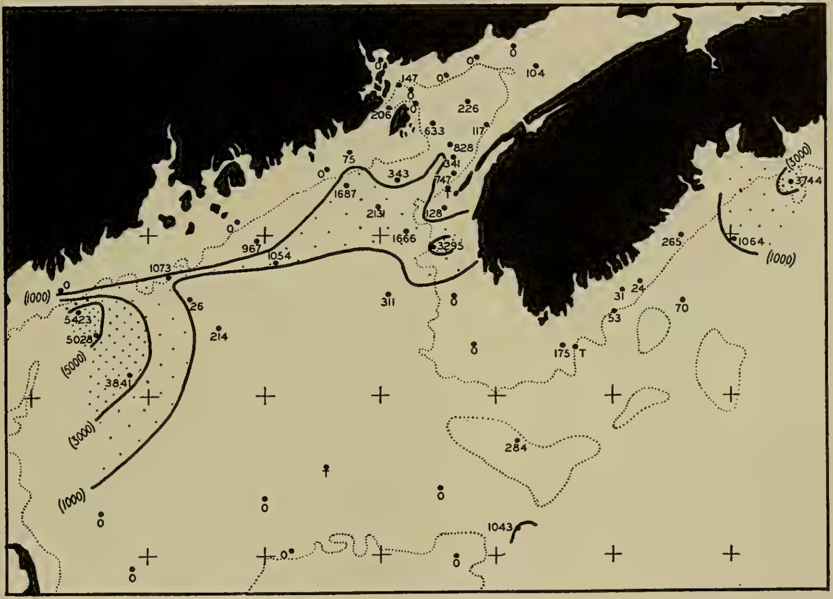
[1] Contribution No. 109.

[2] *Microsetella norvegica,* the second species, is far less important numerically.

In 1931–32 *O. similis* was taken to some extent throughout the Gulf of Maine and Bay of Fundy, the most noticeable changes in the quantitative distribution of the adult stock occurring with the vernal warming of the water. In April, 1932 it was most numerous (up to 5,423 per minute) in the western area of the Gulf with smaller and apparently more local centers of abundance in the Middle Channel, off Yarmouth, and off Halifax. In the outer basin of the Gulf it was almost totally absent in the hauls (Fig. 1).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0
104
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0
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226
633
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828
75
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343
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1687
2131
128
0
(3000)
3744
967
1054
1666
3295
265
1064
(1000)
1073
0
(1000)
5423
5028
26
214
311
0
31
24
70
53
0
175
T
(5000)
3841
(3000)
284
T
(1000)
0
0
0
1043
0
0
0

FIG. 1. Distribution of adults of *Oithona similis* in April, 1932. Number per minute of towing.

The changes in distribution from April to May would suggest that *Oithona* tends to withdraw into deeper water in winter, and approaches the coast with rising temperatures in the spring. By the end of May the greatest number of adults (6,087 per minute) was found at the innermost station off Casco Bay where the surface temperature was 8.63° C., although they were abundant throughout the western area (6.08°–9.38° C.) and in the Bay of Fundy along the course of the entering drift. The richest hauls at this time were everywhere obtained at temperatures exceeding 6° C. *Oithona* was still sparse in the colder waters of the upper Bay, the Quoddy region, and thence westward along the coast of Maine to Frenchmans Bay.

There was a marked increase in the adult stock between May and June but no appreciable changes in distribution. Again the richest hauls were made nearest Casco Bay, and the poorest in the New Brunswick and eastern Maine coastal areas. The adult stock reached its peak at this time in the western Gulf and, although the subsequent crop yielded larger numbers of larvæ in August (Table IV), few apparently reached maturity. East of Mt. Desert adults did not reach their peak until August and the maximum was of short duration for, in the Bay of Fundy at least, they had almost reached the April level by mid-September.

PRODUCTION AND DISPERSAL OF EGGS AND LARVÆ

Spawning Areas

Spawning is widespread but varies greatly in different parts of the region. Until July, in 1932, with the major portion of the *Oithona similis* population located in the western part of the Gulf, the rapid decline to the eastward resembled that of *Calanus finmarchicus* (Fish, 1936*a,* Fig. 11). Only in the outer part of the Bay of Fundy was there a slight break in the descending curve, and here the greatest increase was in copepodite stages, probably transported for some distance from the western or outer Gulf (Fig. 3). This is indicated by the low values in the sections from the inner Bay (*F* and *G*) and in the path of the drift from the Bay along the coast as far west as Mt. Desert (*E*).

The eastern region assumed greater importance in midsummer, and the curve for August (Fig. 3) more closely resembles that of *Pseudocalanus* (Fish, 1936*b,* Fig. 1) than *Calanus.* Extensive spawning continued in the western area, but the large numbers of early and late nauplii entering the Bay of Fundy and eastern Gulf at this time are indicative of important propagation nearby. Early nauplii, up to 19,258 per cubic meter, in the Bay could hardly have originated beyond the eastern basin. This is also evident from the number of early larvæ at all offshore stations east of Penobscot Bay in the Gulf (Fig. 9*C*).

The Bay of Fundy itself, however, appears to be a relatively unimportant spawning area. In the Quoddy region beyond the immediate influence of the drift from the Gulf, no eggs were found before July 30, and at all times both eggs and early stages were comparatively sparse, a condition also prevailing eastward along the New Brunswick coast and at the innermost stations (9, 10*A* and 11*A*). Elsewhere in the

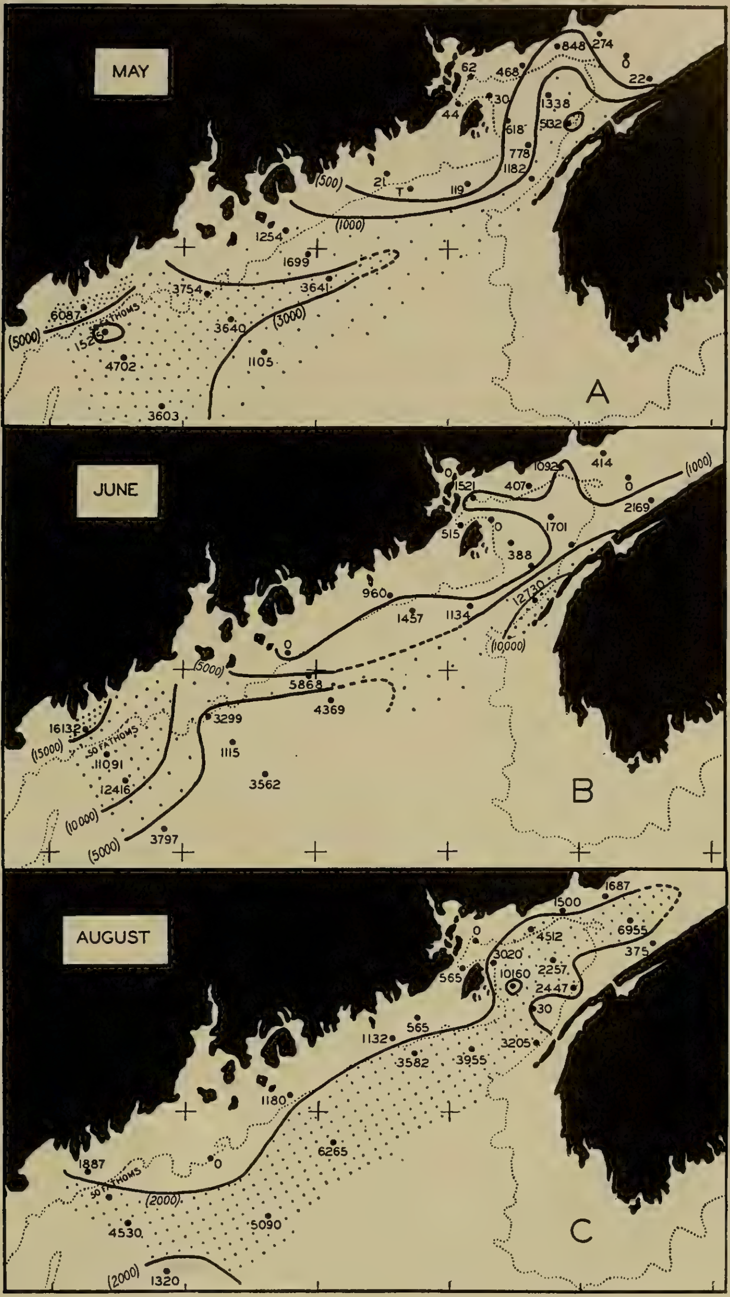

MAY
JUNE
AUGUST
A
B
C
50 FATHOMS
(500)
(1000)
(2000)
(3000)
(5000)
(10,000)
(15000)

FIG. 2. Distribution of adults of *Oithona similis* in 1932. May–June: number per minute. August: number per cubic meter.

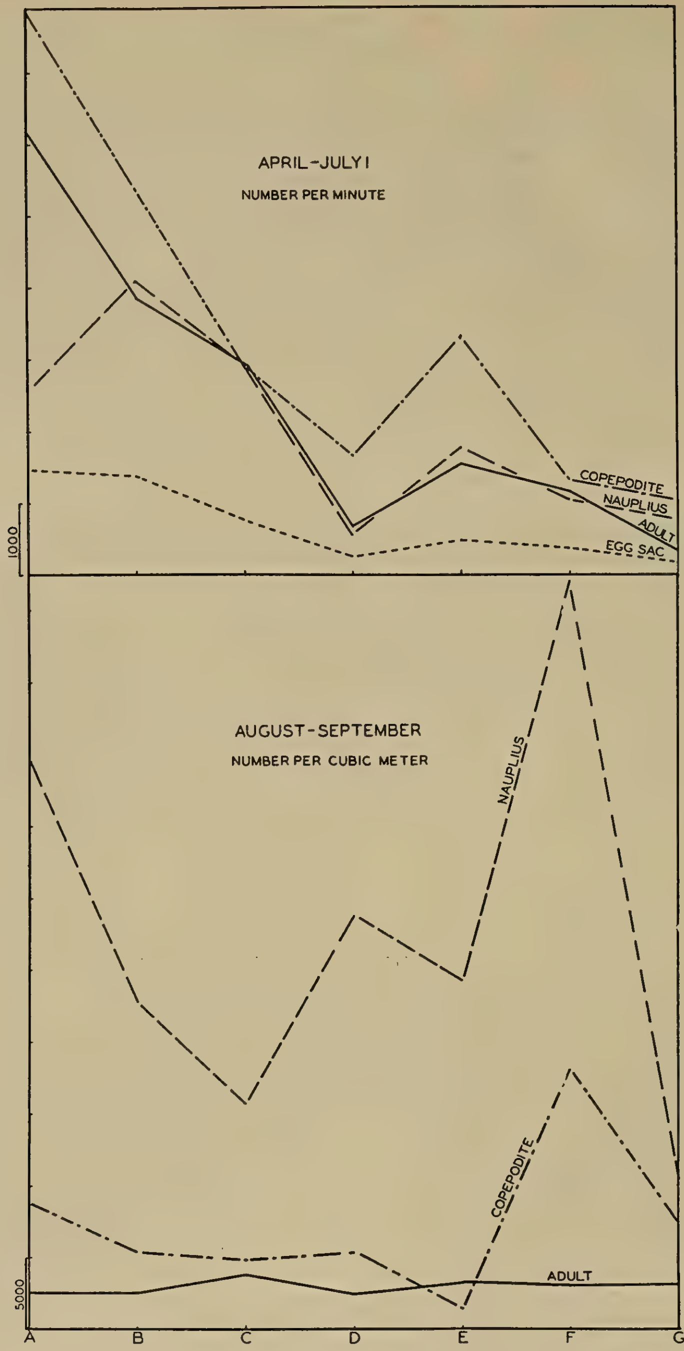


FIG. 3. Mean numbers of adults and young of *O. similis* in seven sections between Casco Bay and Cape Spencer in 1932. Sections: *A*. Casco Bay (Stations 23*A*–25*A*, 26); *B*. Penobscot Bay (Stations 27–29); *C*. Mt. Desert (Stations 17, 30–31); *D*. Moose Peak (Stations 32–33); *E*. Passamaquoddy Bay (Stations 5–8*A*, 35); *F*. Pt. Lipreau (Stations 13, 36–37); *G*. Cape Spencer (Stations 9–11).

Bay they were frequently very abundant, with hauls in August yielding up to 35,970 early nauplii per cubic meter (Fig. 9*C*), but these were always in the path of the entering drift.

Breeding Seasons

Like the other dominant boreal copepods, *Calanus finmarchicus* and *Pseudocalanus minutus, Oithona* begins spawning in the Gulf of Maine in March and continues in a succession of generations until September. There is a seasonal delay of about one month in the appearance of eggs in the eastern Gulf and Bay of Fundy and this regional variation permits the distinguishing of two breeding stocks, a western and an eastern. Also, as in the case of the two above-mentioned species it must be borne in mind that these stocks of *Oithona* are not permanently restricted to their respective localities, but merely represent delayed maturation of that portion of the winter population which happens to be located in the eastern part of the region during the spring, and earlier maturation in areas to the westward where the water mass responds more rapidly to vernal warming. However, since the augmentation starts at significantly different times in the two regions, with substantially the same interval of development, subsequent broods of the two stocks continue to appear at distinct periods irrespective of where they are dispersed.

Annual Cycle in the Two Breeding Stocks

Very little has been published on the biology of this species. Gran (1902) found some indication that the development of small species like *Oithona similis, Microsetella atlantica* and *Oncæa conifera* is completed in a much shorter time than in larger species like *Calanus finmarchicus, C. hyperboreas, Metridia longa,* and *Euchæta norvegica.*

Ruud (1929) reported that the stock of *O. helgolandica* (*similis*) doubled in number off More in 1925 from the end of May to the end of July. In 1926 a small stock at the beginning of March increased steadily until May 12. A second increase took place later, reaching a maximum on July 20. In 1927 conditions were similar except that the second maximum attained the highest peak of the year on July 31. Bogorov (1932) found copepodite stages III and IV predominating in the Barent's Sea at the end of July.

In the Gulf of Maine and Bay of Fundy the maxima of different larval stages, following the appearance of the first brood of the stock maturing in the western Gulf in March, indicate a developmental period throughout the region of about two months during the early season, and about six weeks in summer.

There appear to be at least three and possibly four broods of the western stock in March, May, July, and September. This stock, which in 1932 was represented by a maximum of late nauplius and copepodite

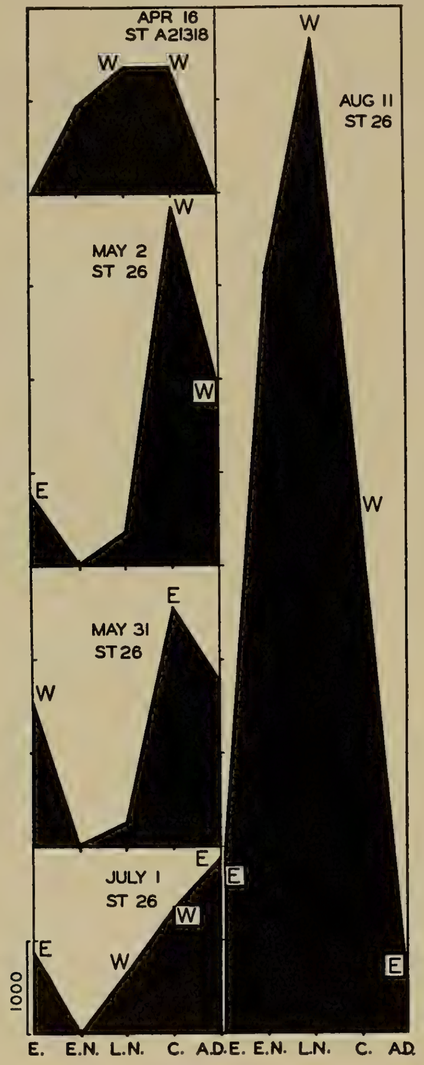


Fig. 4.

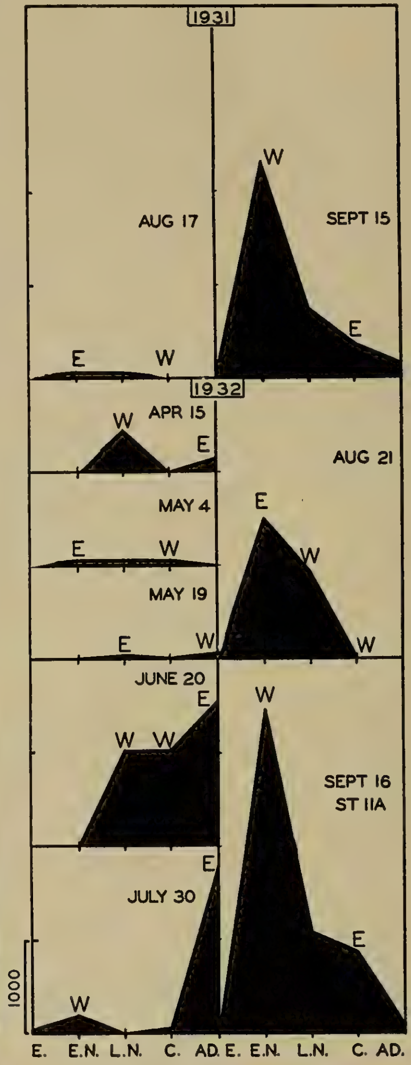


Fig. 5.

Fig. 4. Composition of the population of *Oithona similis* in the outer part of the western area in 1932, showing the succession of generations of the western (*W*) and eastern (*E*) stocks. April–June: number per minute. August–September: number per cubic meter. Stages recorded as *E*-egg sacs, *E.N.*—early nauplius (I–III), *L.N.*—late nauplius (IV–VI), *C.*—copepodites (I–V), *A.D.*—adults.

Fig. 5. Composition of the population of *Oithona similis* at Station 5 in the New Brunswick area of the Bay of Fundy in 1931 and 1932. Symbols as in Fig. 4.

stages on April 16, had by May 2 advanced largely to late copepodite stages and by May 31 matured and was spawning (Fig. 4). The second brood had reached late nauplius-copepodite stages by the end of June. The third brood, apparently developing more rapidly, was dominated by late nauplii in mid-August and what appeared to be a fourth brood was in egg and early nauplius stages on September 15–16 (Fig. 5).

There are three broods indicated in the eastern stock. Eggs found between April 20–May 2 had presumably advanced to copepodite stages by the end of May (Fig. 4). A second brood of eggs appeared a month later, and a third brood by mid-August. That portion of the stock remaining on September 15–16 in the Bay was apparently represented by late nauplius and early copepodite stages.

TABLE I

Dominant stages of the eastern and western stocks of P. minutus and O. similis occurring at comparable periods in 1932.

1932	Western Stock		Eastern Stock	
	P. minutus	O. similis	P. minutus	O. similis
April 16	Late nauplius	Late nauplius-copepodite	Egg	Egg
May 19–22	Late copepodite	Adult	Late nauplius	Late nauplius
May 31	Adult	Egg	Late nauplius-early copepodite	Copepodite
June 20–31	Late nauplius	Late nauplius-copepodite	Egg	Adult-egg
July 30	Egg—early nauplius	Early nauplius	Adult	Adult
August 11	Late nauplius	Late nauplius-copepodite	Adult-egg	Adult-egg
September 15–16	Egg	Egg—early nauplius	Late nauplius	Late nauplius-copepodite

The successive generations of the two breeding stocks of *Oithona* as shown in Table I and in Figs. 4 and 5 compare very closely with those of *Pseudocalanus* (Fish, 1936*b*, Figs. 2–4).

It will be noted that in the western stock *Oithona* in 1932 appeared to be slightly more advanced than *Pseudocalanus* at comparable periods throughout the summer, while in the eastern stock very little difference was detectable.

Productivity and Mortality in Developing Larvæ

Oithona similis was frequently taken during the breeding periods with one or two attached ovisacs. However, by far the larger number was found floating free in the water. Whether or not the sac normally remains attached to the female for only a short period of time to insure fertilization, and then becomes detached to permit the formation of others, is not known. It is, of course, possible that those in the samples were broken off in the process of capture, but considering the large number of larvæ produced by a relatively small parent stock in August, several sacs of eggs must have been produced by each female at that season. Unlike *Pseudocalanus,* where the ovisac was never found around detached eggs, the ovisac of *Oithona* remains intact during the period of incubation and no doubt serves to protect the eggs from small predatory forms until they have hatched, a condition also found in *Microsetella.*

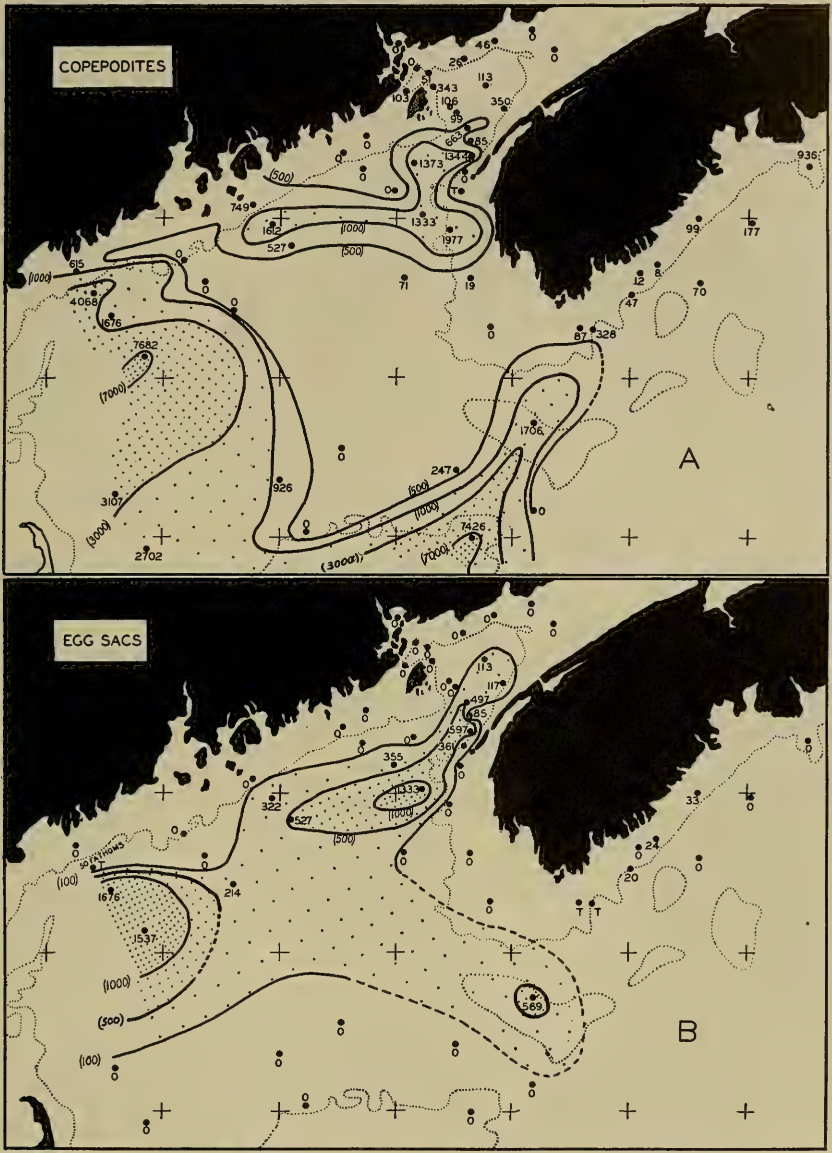
From April until the end of June in 1932 the stock of *Oithona* increased as shown in Table II, but considering the relative number of adults and young, it does not appear to have been an unusually prolific species during this period. In midsummer, however, with the maturing of the second western brood, a third crop of larvæ appeared, outnumbering the combined young of all other species of copepods both in 1931 and 1932 (Tables II and IV). It is not possible to say whether the parent stock of the third eastern brood is equally productive, because spawning had just started at the time of the last cruise in the Gulf, in mid-August.

TABLE II

Mean numbers of larvæ and adults of Oithona similis in the total region. April–June: number per minute. August–September: number per cubic meter.

	Month	Larvæ	Adults
1932	April	1,112	879
	May	5,918	2,519
	June	8,919	3,387
	August	34,852	2,938
	September	3,262	332
1931	August	33,925	3,550
	September	4,825	94

Like *Pseudocalanus,* the greatest mortality in developing larvæ of *Oithona* seems to take place in the nauplius stages. This can be seen by comparing the abundance of nauplii in August with that of copepodites and adults in September (Fig. 9 and Table II). In the Bay of Fundy, where the mean number of late nauplius and copepodite stages in August amounted to 26,655 per cubic meter, September collections showed a decline to 1,720 late larvæ and 332 adults per cubic meter. A similar decline was also found in September, 1931.


COPEPODITES
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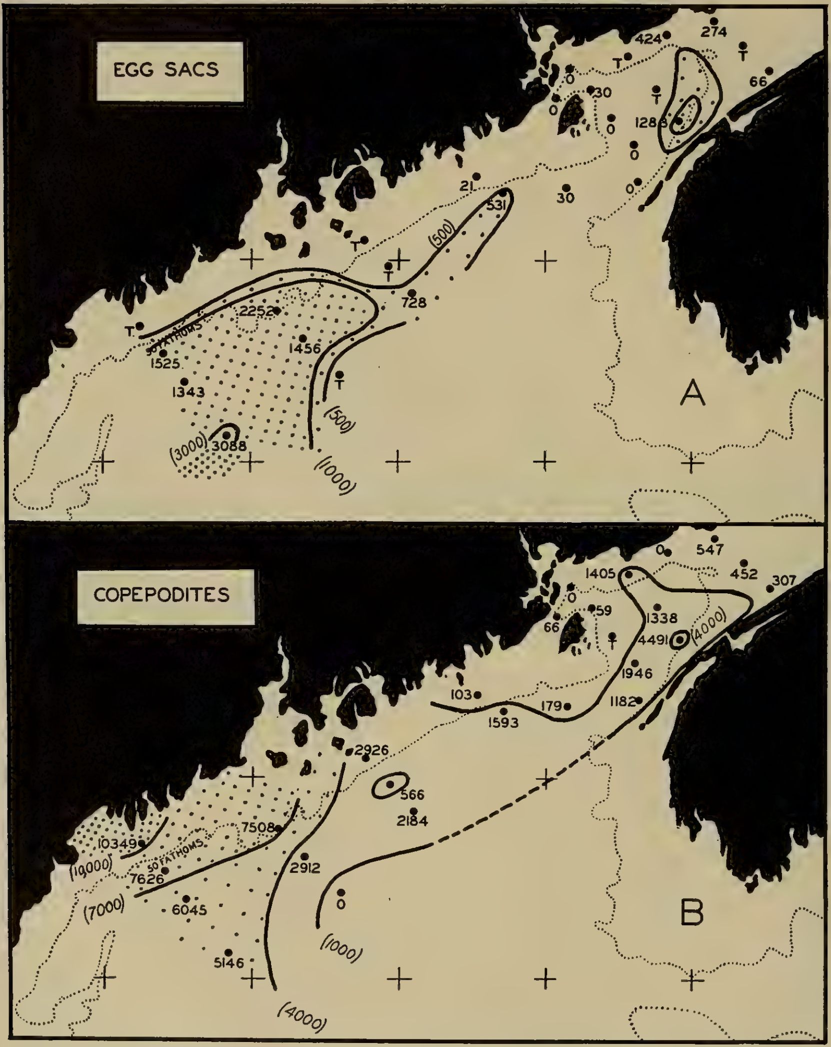
26
113
51
343
103
106
350
99
663
85
1344
1373
(500)
936
749
1612
(1000)
1333
527
(500)
1977
99
177
615
(1000)
71
19
12
8
47
70
4068
1676
87
328
7682
(7000)
1706
A
247
926
3107
(500)
(1000)
(3000)
7426
2702
(3000)
(7000)
EGG SACS
113
117
497
85
597
361
355
1333
322
527
(1000)
(500)
33
24
20
60 FATHOMS
(100)
1676
214
T
T
1537
(1000)
569
(500)
(100)
B

FIG. 6. Occurrence of copepodite stages of the western stock and egg sacs of the eastern stock in April, 1932. Number per minute of towing.

Dispersal and Regional Abundance of Eggs and Larvæ

Dispersal in 1932.—The method of tracing dispersal by monthly changes in the distribution of developmental stages of the different breeding stocks has been used in the present analyses. The general surface circulation and expected drift of eggs and larvæ within the Gulf and Bay of Fundy has previously been described (Fish, 1936*b*).


EGG SACS
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274
T
T
66
0
30
0
128
0
0
0
21
531
30
(500)
T
T
728
2252
T
50 FATHOMS
1525
1456
T
1343
A
(500)
(3000)
3088
(1000)
COPEPODITES
0
547
1405
452
307
0
59
66
1338
4491
(4000)
1946
103
179
1182
1593
2926
566
2184
7508
10349
50 FATHOMS
(10,000)
7626
2912
(7000)
6045
0
B
(1000)
5146
(4000)

FIG. 7. Occurrence of egg sacs representing the second brood of the western stock and copepodite stages of the first brood of the eastern stock in May, 1932. Number per minute of towing.

In the case of *O. similis* there was an apparent difference in the general distribution of larvæ of the two breeding stocks. Spawning of the western stock began seaward of the 100-meter contour and subsequent generations remained for the most part concentrated offshore. The eastern stock, on the other hand, although emanating largely from the eastern basin, soon became widely dispersed in the coastal zone, and succeeding broods, particularly in the more advanced larval stages (Fig. *7B*), were until August at times more abundant inside than outside of the 100-meter contour.

Western Stock.—Spawning offshore in the western basin and in the region of Georges Bank in March, by April 16 (Fig. 6*A*) larvæ in late nauplius and copepodite stages were found most abundant in the offing of Casco Bay and seaward in an arc corresponding with the general surface drift to the outer banks. Smaller numbers occurred in the eastern basin, the Bay of Fundy and along the south coast of Nova Scotia. Like *Calanus* and *Pseudocalanus,* in outer waters *Oithona similis* appears to reproduce off Halifax as early as in the western basin of the Gulf.

This brood remained centered in the western area, although by late May when the stock had matured and was spawning (Figs. 2*A* and 7*A*), a considerable number, having presumably circled the outer Gulf, were found entering the Bay of Fundy along the Nova Scotian side. The largest single haul of adults (possibly eastern stock; see p. 181) was made at the innermost station off Casco Bay, but egg sacs were for the most part concentrated offshore westward from Mt. Desert.

The second brood greatly increased the size of the western stock and by the end of June had become generally distributed offshore in the Gulf. The larvæ, at this time in late nauplius and copepodite stages (Fig. 8*A*), were abundant in all hauls seaward of the 100-meter line, particularly in the Casco section and along the Nova Scotian side of the Bay of Fundy. The fact that the numbers of both late nauplii (17,938 per minute) and copepodites (32,404 per minute) at the entrance to the Bay (Station 8*A*) exceeded those found anywhere in the inner Gulf may indicate that immigrants from the western area are supplemented in June by contributions from another important production area, probably in the vicinity of the outer banks.[3]

Once the second brood had become generally dispersed offshore very little change was noted thereafter in the relative regional abundance of comparable stages during the remainder of the summer. In mid-August copepodites of the third and largest brood (Fig. 9*B*) were distributed in much the same manner as similar stages of the preceding brood in June (Fig. 8*A*), being abundant at all offshore stations. Late nauplii of the same brood (Fig. 9*A*) were somewhat more restricted, with concentrations west of Penobscot Bay and in the drift entering the Bay of Fundy.

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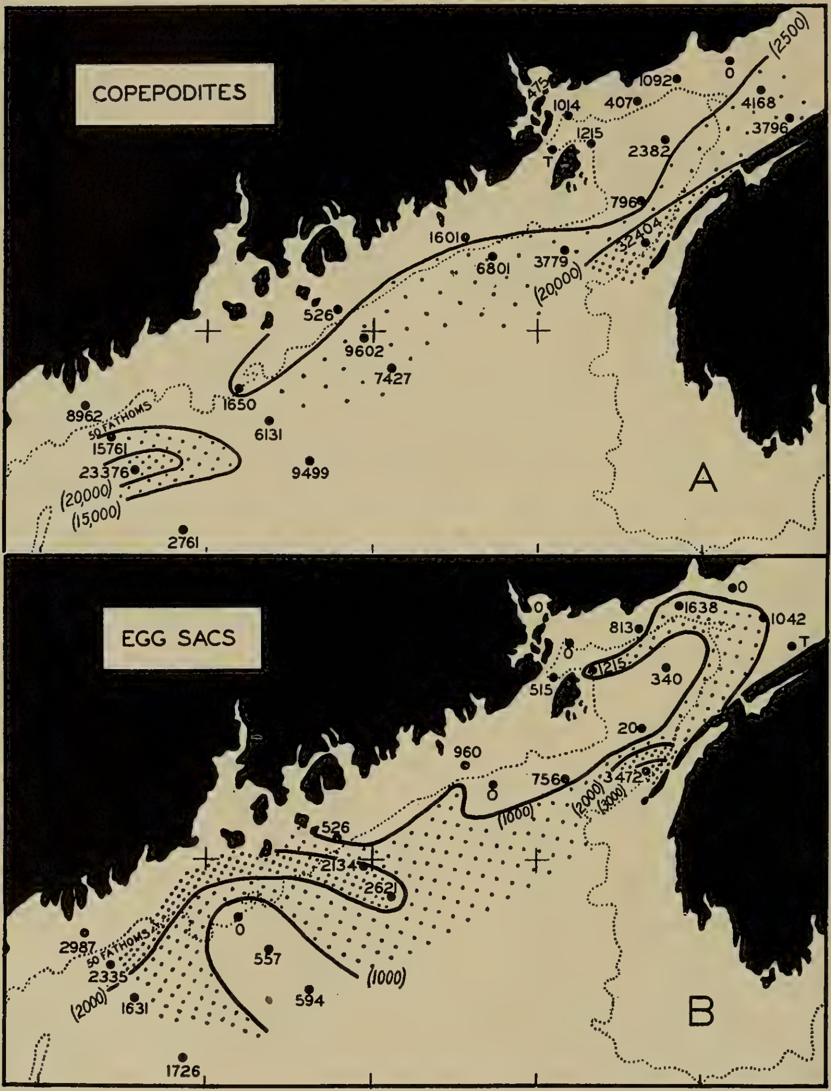
[3] Since immigrant larvæ of other species, abundant at the time, showed no evidence of unusual concentration at Station 8A (Fish, 1936*a* and *b*), this condition in the case of *Oithona* does not appear to have been due to local current action.


COPEPODITES
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1014
1092
407
0
(2500)
4168
3796
1215
T
2382
796
32404
1601
6801
3779
(20,000)
526
9602
7427
1650
8962
50 FATHOMS
15761
23376
(20,000)
(15,000)
6131
9499
2761
A
EGG SACS
0
0
1638
1042
813
T
0
1215
340
515
20
960
756
3472
(2000)
(3000)
(1000)
0
526
2134
2621
0
2987
50 FATHOMS
2335
(2000)
1631
557
594
(1000)
1726
B

FIG. 8. Occurrence of copepodites of the second brood of the western stock and egg sacs of the second brood of the eastern stock in June, 1932. Number per minute of towing.

The coastal area east of Penobscot Bay and including the Bay of Fundy would appear to be relatively unproductive. The Bay apparently derives at times an abundant supply of nauplius stages from the eastern basin (Fig. 9*C*), but the eastern coastal region of the Gulf was found to be populated for the most part with a relatively small immigrant stock in more advanced stages (copepodites and adults). The large numbers of nauplii entering the Bay of Fundy along the Nova Scotian side must decline rapidly in the cold turbulent waters for there is little evidence that they survive the circuit of the Bay and are dispersed westward in the drift along the coast of Maine. The most probable source of the late stages found in the latter zone, east of Mt. Desert, is that portion of the drift from the outer Gulf and eastern basin passing westward across the entrance to the Bay.

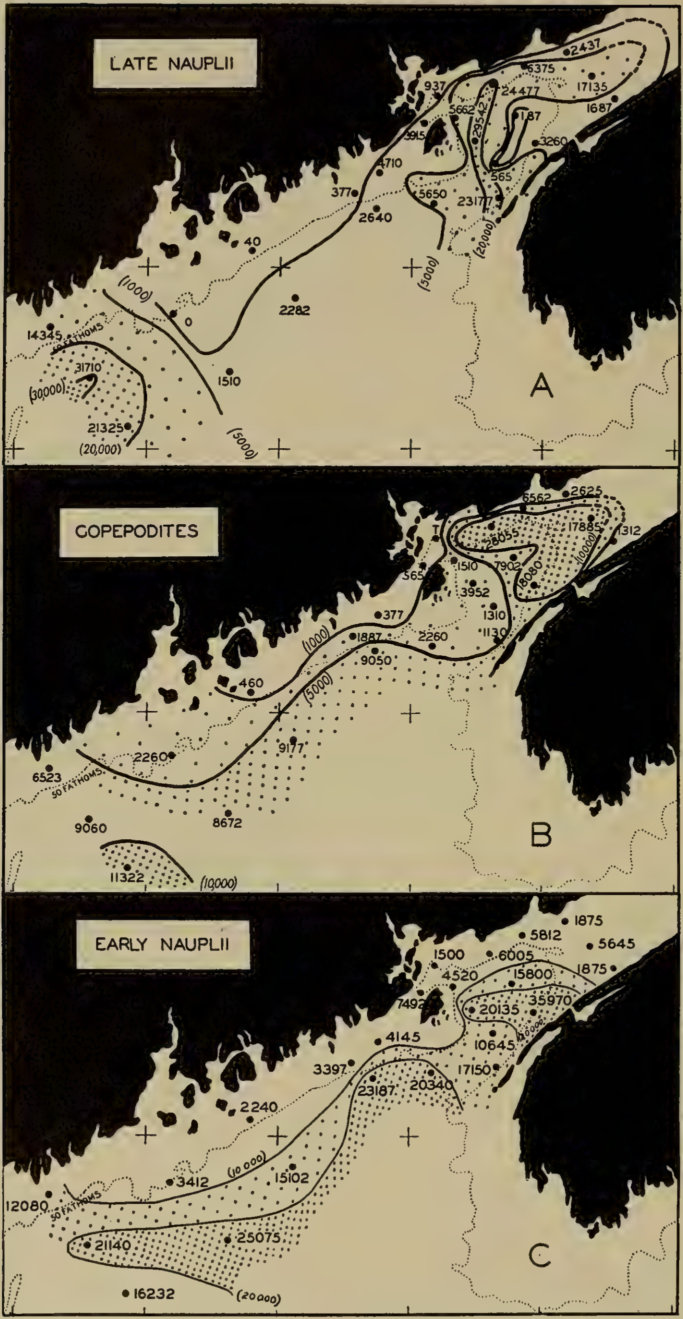
By mid-September the *Oithona* population had greatly declined in the Bay of Fundy. Observations were not made in the Gulf after August, but in view of the absence of an appreciable influx in the entering drift it is probable that the Bay reflected conditions throughout the region.

Eastern Stock.—The first brood of the stock maturing in the eastern part of the inner Gulf and Bay of Fundy was just appearing near its southern and western margins in late April. Eggs were found in the eastern basin, over German Bank, and in a limited portion of the western basin, but where vernal warming was delayed in the more turbulent eastern coastal zone of the Gulf and the Bay there was as yet no evidence of propagation. Egg sacs west of Mt. Desert were all found in the drift from the eastward and were probably derived from a parent stock transported from the eastern basin (Fig. 6*B*). No eggs were found in the coastal zone anywhere in the Gulf.

By late May this first brood consisted largely of late copepodite stages which had become generally dispersed both in inshore and offshore waters, being most numerous well inshore in the western area (Fig. 7*B*). Some in the warmer western coastal zone had apparently passed through the final moult, for as indicated by the scarcity of eggs (Fig. 7*A*) at the innermost station off Casco Bay (Station 25*A*) adults there (Fig. 2*A*) were sexually immature and probably represented for the most part members of the eastern stock.[4]

Further evidence that only those eggs transported beyond the eastern coastal region before hatching survive, is seen in the composition of the eastern stock at this time. Since propagation had not begun in the inner Gulf east of Mt. Desert by the end of April, the product of this

[4] The western stock offshore had already spawned at this time (Fig. 7*A*).


LATE NAUPLII
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6375
24477
17135
1687
937
5662
29542
187
3260
3915
4710
565
377
5650
23177
2640
(20,000)
40
(5000)
(1000)
2282
0
14345
50 FATHOMS
31710
(30,000)
1510
21325
(20,000)
(5000)
A
COPEPODITES
6562
2625
17885
1312
28055
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1510
7902
565
3952
18080
1310
377
1130
1887
2260
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9050
460
(5000)
9177
2260
6523
50 FATHOMS
9060
8672
B
11322
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1875
5812
5645
1500
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7492
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20135
4145
10645
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3397
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23187
20340
2240
(10 000)
15102
3412
12080
50 FATHOMS
21140
25075
16232
(20000)
C

FIG. 9. Occurrence of late nauplii and copepodites of the third brood of the western stock, and early nauplii of the third brood of the eastern stock in August, 1932. Number per cubic meter.

late seasonal spawning a month later should have been represented by late nauplius stages, but very few were found. The young occurring in this region were copepodites assumed to be immigrants from more favorable production areas.

The distribution of eggs of the second eastern brood in late June (Fig. 8*B*) corresponded closely to that of the parent stock in the previous month (Fig. 7*A*). They were most numerous at the innermost stations in the Gulf and along the course of the drift into the Bay.

In mid-summer *Oithona* appeared to withdraw from the coast and the third eastern brood, in egg and nauplius stages, was found in August centered in the eastern basin and extending eastward into the Bay of Fundy and westward along the outer stations to the western area (Fig. 9*C*).[5]

TABLE III

Mean numbers of Oithona similis in the Gulf of Maine in 1931 and 1932. Number per cubic meter.

Stage	Gulf of Maine			
	Western Area		Central Area	
	1931 (Aug. 21–25)	1932 (Aug. 11–14)	1931 (Aug. 26)	1932 (Aug. 8–15)
Egg sac	1,947	1,855	1,409	1,224
Early nauplius	11,765	15,363	8,044	10,981
Late nauplius	12,018	15,529	21,133	11,175
Copepodite	7,908	7,217	9,220	5,144
Adult	3,537	2,557	3,575	3,090

As in the case of the western stock, there must have been a great decline in the third eastern brood in late summer. In the Bay of Fundy by mid-September the survivors in late nauplius and copepodite stages were found entering along the Nova Scotian side in numbers not exceeding 3,000 per cubic meter.[6]

Annual Variation.—In the Gulf the mean numbers of larvæ and adults in August of the two years were of a somewhat similar order. In 1931 (August 21–26) there were 35,044 larvæ and 3,556 adults per cubic meter, and in 1932 (August 8–15), 32,705 larvæ and 2,824 adults. However, the mean for the Bay of Fundy at the beginning of September in 1931 was more comparable with that of mid-September than of mid-August of the following year. In 1932 larvæ declined from 37,716 on August 17–21 to 3,262 per cubic meter on September 15–16, and adults from 3,120 to 332. The decrease had apparently already taken place in the Bay by September 1–5 in 1931, when the mean number of larvæ, 4,826, was almost as low as in the previous year in mid-September and the number of adults, 94, even lower.

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[5] Within the region of observations in the Gulf in 1932, copepodites of the eastern stock in May (Fig. 7*B*) and eggs in June (Fig. 8*B*) were most abundant in the coastal zone westward from Mt. Desert.

[6] In August in this area early nauplii were found up to 35,970 per cubic meter.

Considered by areas, in the Gulf the total numbers and relative percentages in comparable stages exhibit no very striking differences (Table III). A similar condition in the case of *Calanus* (Fish, 1936*a*, p. 130) and *Pseudocalanus* (Fish, 1936*b*, p. 202) has been interpreted to mean that in 1931 the season was delayed about two weeks. Adults were slightly more numerous in both the central and western areas in 1931. The mean number of larvæ was higher in the western area in 1932, and in the central area in 1931.

TABLE IV

Mean numbers of larval stages of dominant species of copepods in the total region in 1932. April–June: number per minute. August–September: number per cubic meter.

1932	Calanus finmarchicus	Pseudocalanus minutus	Oithona similis
April	2,007	7,126	1,112
May	7,024	11,484	5,918
June	2,156	21,303	8,919
August	977	5,639	34,852
September	41	3,506	3,262
April–Sept.	(Mean) 2,441	9,812	10,813

RELATIVE NUMERICAL STRENGTH OF O. SIMILIS IN THE ZOÖPLANKTON POPULATION

Owing to its small size, *Oithona similis* is not taken in representative numbers in plankton nets of the type usually used in American waters and for this reason former available records do not indicate accurately its relative abundance in the boreal population of the western Atlantic. Its presence in large numbers in collections from the Newfoundland region whenever there was a considerable amount of ctenophore debris (Pinhey, 1926) would indicate that it is abundant in those waters in late summer, but was captured only when the meshes of the net became partially clogged. As far south as Chesapeake Bay *O. similis* has been found by Wilson (1932) to form an important addition to the winter plankton. In the eastern Atlantic Gran (1902) reported that this species also plays an important rôle off Norway, and Ruud (1929) in the same region found it comprising up to 60 per cent of the copepod stock in July, 1925.

In the present investigations *Oithona similis* rarely appeared in the coarse meter net samples, but an estimate of its importance in the region during the spring and summer months can be obtained by comparing the relative abundance of larvæ with those of other dominant species. In making such a comparison it must be borne in mind that the relative numbers of young do not necessarily indicate that adults occur in the same proportion. The mortality of developing young appears to be very much greater in some species than in others and, like *Pseudocalanus* (Fish, 1936*b*), a very small percentage of *Oithona* larvæ apparently reach maturity. Again, because of its small size *Oithona* in the adult form must rank far below *Calanus* and *Pseudocalanus* in importance. However, it is not the adult stock of *Oithona* but the tremendous numbers of larvæ produced by this prolific species which appears to be of greatest significance in the economy of the region. Particularly in August it must form a most important source of food for those species feeding on larval copepods. In Table IV it will be seen that, based on the mean numbers of young between April and September (1932), *Oithona* ranks with *Pseudocalanus* and far exceeds *Calanus*.

Of added importance in the economy of the region is the fact that the seasonal maxima of the three species occur at successively later periods during the summer. Whereas *Calanus* reaches its peak in May (first brood), *Pseudocalanus* is most abundant in June (second brood), and *Oithona* (third brood) in August.[7] In the latter month the mean of *Oithona* larvæ for the entire region amounted to 34,852 per cubic meter.

SUMMARY

1. *Oithona similis,* although almost world-wide in distribution, is centered largely north of Cape Cod on the western Atlantic coast, and south from this point is gradually replaced by *O. brevicornis*.

2. The adult stock in the Gulf of Maine appears to approach the coast from offshore with rising temperatures in the spring.

3. Propagation begins in the Gulf in March and continues in a succession of generations until September.

4. Two breeding stocks are distinguishable, due to an average difference of about one month in the time of vernal propagation in the

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[7] To this seasonal progression may be added a September maximum of *Centropages typicus* larvæ throughout the Gulf and Bay of Fundy.

eastern and western parts of the region. This difference is reflected in subsequent generations after dispersal.

5. There appear to be four broods of the western stock, in March, May, July, and September, and three in the eastern, in April, June, and August.

6. A developmental period of two months during the early season and about six weeks in summer is indicated.

7. *Oithona* is at times far more prolific than either *Calanus* or *Pseudocalanus,* but mortality is relatively much higher, depletion being greatest in nauplius stages.

8. The western stock remained for the most part concentrated offshore, but the eastern, particularly in advanced stages, was more generally distributed, sometimes being more abundant inside than outside of the 100-meter contour.

9. There is some evidence that in June the local stock in the inner Gulf is supplemented by important contributions from another production area, probably in the vicinity of the outer banks.

10. There is no indication of successful propagation in the turbulent coastal region of the Gulf east of Mt. Desert or in the Bay of Fundy.

11. The larvæ of *Oithona* afford the most abundant source of food in the region in mid-summer for those animals feeding on larval planktonic organisms. Of added importance in the natural economy of the region is the fact that the seasonal maxima of the three numerically dominant species occur at successively later periods during the summer, *Calanus* in May, *Pseudocalanus* in June, and *Oithona* in August.

BIBLIOGRAPHY

Bigelow, H. B., 1926. Plankton of the offshore waters of the Gulf of Maine. *Bull. U. S. Bur. Fish.,* vol. 40, Part II, 1924 (1926). Washington.

Bogorov, B. G., 1932. Materials on the biology of copepods of the Barent and White Seas. *Bull. State Oceanographic Institute.* Moscow. Part 4.

Fish, C. J., 1925. Seasonal distribution of the plankton of the Woods Hole region. *Bull. U. S. Bur. Fish.,* **41:** 91, 1925 (1926). Washington.

Fish, C. J., 1936*a*. The biology of Calanus finmarchicus in the Gulf of Maine and Bay of Fundy. *Biol. Bull.,* **70:** 118.

Fish, C. J., 1936*b*. The biology of Pseudocalanus minutus in the Gulf of Maine and Bay of Fundy. *Biol. Bull.,* **70:** 193.

Gran, H. H., 1902. Das Plankton des Norwegischen Nordmeeres von biologischen und hydrografischen Gesichtspunkten behandelt. Report on Norwegian Fishery and Marine Investigations, Vol. II, Part II (1909), No. 5. Bergen.

Herdman, W. A., I. C. Thompson, and A. Scott, 1898. On the plankton collected continuously during two traverses of the North Atlantic in the summer of 1897; with descriptions of new species of Copepoda; and an appendix on dredging in Puget Sound. *Proc. and Trans., Liverpool Biol. Soc.,* vol. 12, Session 1897–98 (1898), p. 33. Liverpool.

PINHEY, K. F., 1926. Entomostraca of the Belle Isle Strait Exposition, 1923, with notes on other planktonic species. *Contr. Canad. Biol.,* N. S., vol. 3, No. 6. Ottawa.

RUUD, J. T., 1929. On the biology of copepods off Møre, 1925–1927. *Rapports et Procès-Verbaux, Conseil perm. internat. pour l'exploration de la mer,* vol. 56.

SCOTT, T., 1905. On some Entomostraca from the Gulf of St. Lawrence. *Trans. Nat. Hist. Soc. Glasgow,* vol. 7, N. S., Part I, 1902–1905 (1907), p. 46.

WILSON, C. B., 1932. The copepod crustaceans of Chesapeake Bay. *Proc. U. S. Nat. Museum,* vol. 80, No. 2915. Washington.

THE EFFECT OF ULTRACENTRIFUGING UPON CHICK EMBRYONIC CELLS, WITH SPECIAL REFERENCE TO THE "RESTING" NUCLEUS AND THE MITOTIC SPINDLE[1]

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Few, if any, problems of the cell are more perplexing to the cytologist than those of the structure of the "resting" nucleus and of the mitotic figure. Some of the structures involved can be seen in the living cell, but for others we must rely almost exclusively upon fixed and stained preparations for our information. Because of this fact, marked difference of opinion exists concerning those structures that can only be seen in the fixed preparations; many investigators consider them artifacts which have no counterpart in the living cell.

Within the past four years, work in this laboratory with the ultracentrifuge has to some extent concerned itself with the problems of the physical nature of protoplasm, particularly its components and inclusions in certain somatic tissues (Beams and King, 1934*a*, *b*; 1935). In the present study we propose to limit our description mainly to the effects of ultracentrifuging upon the "resting" nucleus, chromosomes, and mitotic apparatus, hoping thereby to contribute to the knowledge of their physical constitution.

MATERIAL AND METHODS

The method of centrifuging is, for the most part, similar to that already described (Beams and King, 1934*a*). Forty-eight to 96-hour-old chick embryos were removed from the egg and placed in physiological salt solution warmed to 102° F. They were then immediately transferred in the physiological solution to the rotor of the air-driven ultracentrifuge, where they were whirled for 10 minutes at approximately 150,000 times the force of gravity and immediately thereafter fixed in Bouin's solution. Sections were cut and were stained in Delafield's or in Heidenhain's hematoxylin.

DESCRIPTION

In Fig. 1 is shown a mesenchyme cell of the sclerotome region of an 80-hour chick embryo, illustrating the usual position of the inter-

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[1] Aided by grant from the Rockefeller Foundation for work on cellular biology.

kinetic or "resting" nucleus and the normal distribution of its various components, namely, nucleoli, chromatin, "reticulum," nuclear "sap" (karyolymph) and nuclear membrane. It is quite evident that in the nuclei of these, unlike the condition for other cells (Herrick, 1895; Gray, 1927), the distribution of the various components is not noticeably affected by the normal pull of gravity. However, when the force of gravity is increased 150,000 times, a marked displacement and stratification of the nuclear materials in the order of their relative specific gravity is evident, as illustrated in Figs. 2, 3, 4, and 5. In Fig. 2 the nucleolus is shown displaced to the heavy or centrifugal pole, the chromatin (basichromatin) and "reticulum" or "linin" (oxychromatin) materials are next in order and the non-staining nuclear "sap" or karyolymph is concentrated at the lighter or centripetal pole of the nucleus. In this preparation the nucleus as a whole has not been greatly displaced within the cell. However, in Figs. 3, 4, and 5, a progressively more extensive effect is evident in that the nucleus is stretched in a direction parallel with that of the centrifugal force. The condition in Fig. 5 is extreme, as the nucleus has been extended to over four times its normal length; this illustrates beautifully the elastic properties of the nuclear membrane and the materials included in it. We have never encountered in any of our experiments evidence to support the view that the nucleus is anchored in position by cytoplasmic strands from the cell membrane, as has been figured for cells from the hair of the squash by Heidenhain (1907).

A somewhat different effect is seen in the resting nucleus of the red-blood corpuscle, where a separation of the chromatic from the achromatic elements of the nucleus is effected (Fig. 10). The darkly staining mass at the centrifugal pole contains the chromatic moiety while the rounded clear body at the opposite or centripetal pole is the achromatic or nuclear "sap" portion.

This condition has been noted by us in centrifuged paramecium (King and Beams, 1934): such animals have been observed to live for several days, but apparently do not divide normally (unpublished data). This phenomenon clearly demonstrates that, in these cells too, the chromatic elements of the nucleus are distinctly heavier and the achromatic materials lighter than the cytoplasm.

In reference to the spindle in the following description of dividing cells it is evident that we are dealing with what Schrader (1934) has termed half-spindle and continuous fibers. However, since our material is not favorable for a careful differentiation of these components, we are not here concerned with the origin of the spindle components but will simply speak of them collectively as spindle fibers.

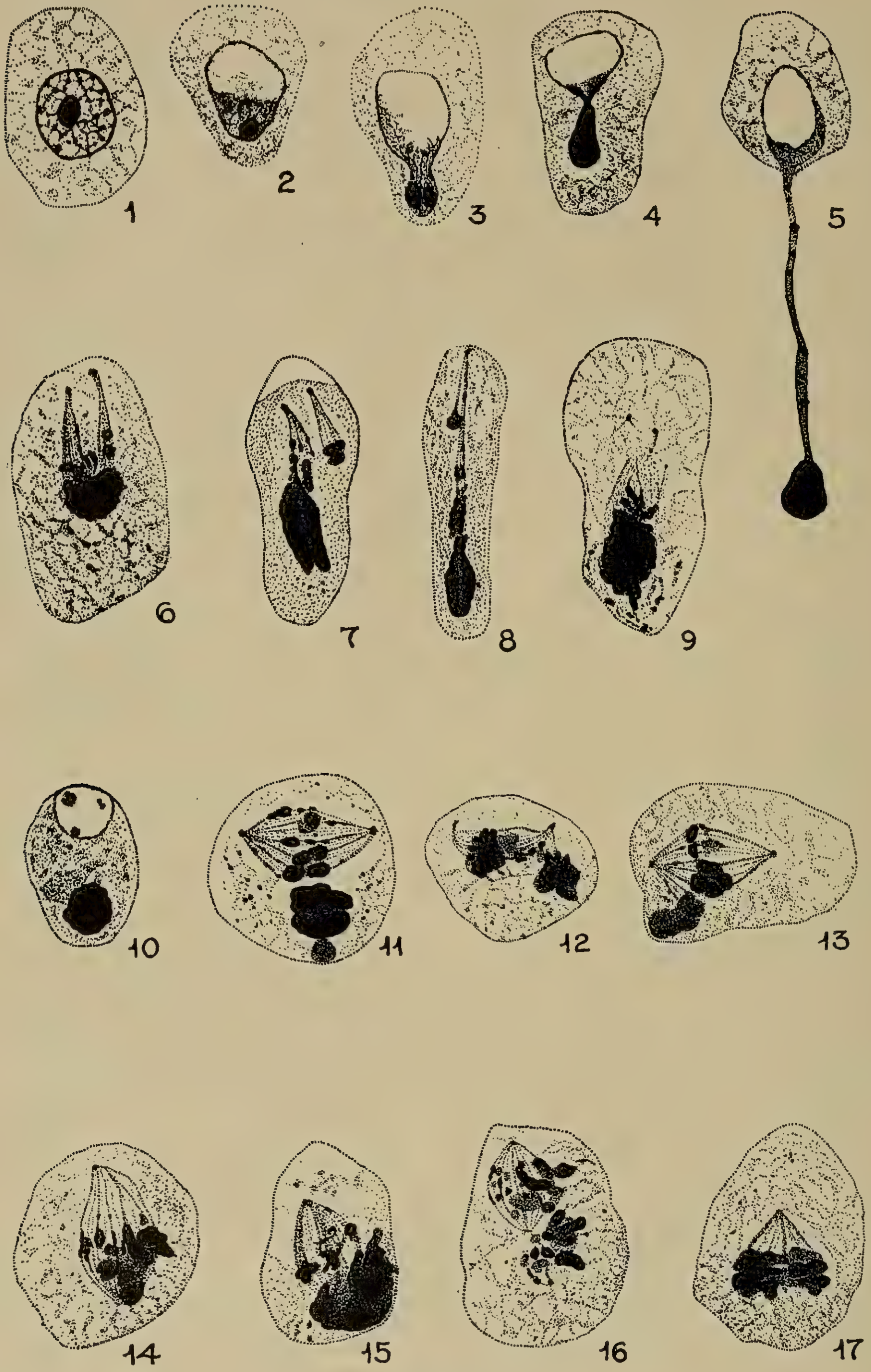
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PLATE I

Many different effects of centrifugal force upon the metaphase mitotic spindle are noted, depending to a large degree upon the orientation of the spindle as regards the direction of the force. For instance, in Figs. 6, 7, 8, and 9, the force has been applied at right angles to the spindle and rather marked effects are observed. In Fig. 6, instead of the mitotic spindle being displaced *in toto* through the cytoplasm of the cell as is often the case in other material (Lillie, 1909; Spooner, 1911; Morgan, 1927), the two halves of the spindle with their associated centrioles are often stretched and bent centripetally through an angle of approximately ninety degrees; the attached metaphase chromosomes in this particular case have not been greatly displaced, nor has the spindle returned to its normal position after release of the centrifugal force. However, in Figs. 7, 8, and 9, similar but somewhat more marked effects upon the spindle are noted. In Figs. 7 and 8 the bulk of the chromosomes has been displaced centrifugally and torn free from one half of the spindle; the other half of the spindle is greatly stretched and apparently was in the process of separating from the chromosomes when the centrifuge was stopped. It is of considerable interest that after such treatment the detached halves of the spindle still possess well-

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EXPLANATION OF FIGURES

All the drawings were made by Miss Gladys Larsen from mesenchyme cells of approximately 80-hour-old chick embryos unless otherwise indicated. The figures have been magnified approximately 2,000 times. The direction of the centrifugal force in all cases has been toward the bottom of the plate.

FIG. 1. Control cell showing the usual distribution of the "resting" nuclear elements.

FIGS. 2, 3, 4, and 5. Centrifuged cells showing a progressively more drastic effect upon the "resting" nucleus and illustrating the relative specific gravity and elastic properties of its materials.

FIGS. 6, 7, 8, and 9. Cases illustrating the bending and stretching of the spindle toward the centripetal pole, displacement of the chromosomes from the metaphase plate centrifugally and disintegration of the spindle, leaving the centrioles free in the cytoplasm.

FIG. 10. Blood corpuscle. The nucleus has been separated into a chromatic portion (at the centrifugal pole) and an achromatic portion (at the centripetal pole).

FIGS. 11, 12, and 13. Cases depicting the displacement of portions of the chromosomes from the spindle. Always a portion of each chromosome remains attached to the spindle fibers. Separation of some of the spindle fibers are also observed.

FIG. 14. Cell showing displacement of the chromosomes along the centrifugal half of the spindle.

FIG. 15. Cell showing displacement and apparent disintegration of the centrifugal half of the spindle.

FIG. 16. Cell with its spindle displaced to the centripetal pole. Portions of the chromosomes have been torn from the spindle.

FIG. 17. Cell with an apparently normal centripetal half of a spindle, but with the lower centrifugal half missing.

defined centrioles and spindle fibers. Here, as in other cases to be mentioned later, fragments of the chromosomes still remain attached to the spindle fibers; this definitely indicates that the union between spindle substance (fibers) and chromosomes is stronger than the internal cohesion of the chromosomes. Furthermore, it is of interest that, when they are free of most of their chromosome material, the half spindles move readily to the centripetal pole of the cells, and hence must be lighter than the surrounding cytoplasm. In the final stages the entire achromatic spindle, save for the centrioles, frequently undergoes a marked physical disintegration (Fig. 9). Only faintly can one observe a portion of the spindle fibers on the centripetal side of the chromosome mass. The two centrioles seem to be completely detached from the spindle substance and are free to move in a centripetal direction. They are not, however, as near the centripetal pole of the cell as might have been expected from the condition in Figs. 7 and 8.

In still other material subjected to the same treatment, cells may be found where most of the chromosomes have been thrown off the equatorial plate of the spindle into the centrifugal end of the cell; the spindle remaining apparently normal in the centripetal end of the cell with very small portions of the chromosomes still attached at its equatorial plate (Fig. 11). Probably the spindle has been greatly distorted, as shown in Fig. 6, during the action of the force, and subsequently has returned to normal upon cessation of centrifuging. Frequently, in similarly treated cells, the spindle has been separated, since part of the spindle fibers can be seen to be still attached to the displaced chromosomes (Figs. 12 and 13). The presence of fragments of chromosomes remaining attached to the spindle at the equatorial plate after such treatment strongly suggests that the spindle and the union between the spindle substance and chromosome is able to withstand greater distortion before breaking than the substance of the chromosomes themselves.

When the mitotic spindle is oriented approximately parallel to the centrifugal force, quite different effects are found. In Fig. 14 the force has acted slightly obliquely from the upper left, forcing the bulk of the chromosomes centrifugally and to the right side of the lower half of the spindle. Here too, as in other cases mentioned above, the fiber attachment portions of the chromosomes are not displaced from the equatorial plate. Sometimes most of the chromosomes are thrown completely free from the spindle into the centrifugal half of the cell (Fig. 15). We were unable to determine whether or not the centrifugal portion of the spindle in this instance has disintegrated or is only masked by the displaced chromosomes. In any case, the centripetal half of the spindle seems to be normal and to possess the usual chromosome fragments on the equatorial plate at the points of spindle attachments. Other cells, treated in a like fashion, often have only part of their chromosomes displaced from the spindle (Fig. 16). In such cases the intact spindle, with its remaining chromosomes and fragments of displaced chromosomes, usually moves into the centripetal half of the cell, the displaced chromosomes to the centrifugal half. Finally cells are found which have a normal centripetal half of a spindle with its metaphase plate of chromosomes while the lower or centrifugal half of the spindle is apparently absent altogether (Fig. 17). This condition is strikingly similar to that recently reported for the onion root tip by Luyet (1935). In such a case, the question immediately arises whether the lower half of the spindle has been forced centripetally, as is known to be the case in Fig. 6, and subsequently lost to view, as has been suggested by Luyet; or whether it is no longer distinct from the surrounding cytoplasm, due perhaps to the pressure developed between the chromosomes and the cell membrane. We are inclined to accept the latter view, because we have never seen the lower half of the spindle in the large number of such cases examined. In this connection the observations of Harvey (1935) are of interest: she observed that the mitotic spindle of certain sea-urchin eggs moved to the centripetal pole upon centrifuging and that the astral rays became much shortened on the compressed or centripetal side of the aster.

The question as to the survival of these chick cells has not been satisfactorily answered as yet. We hope that sometime in the near future attempts may be made to grow centrifuged chick cells in tissue cultures. However, we already know that root tip cells of wheat seedlings do recover after similar distortion by ultracentrifuging.[2]

DISCUSSION

Notwithstanding the fact that thousands of papers have appeared describing and illustrating the nucleus in practically every type of cell since it was first discovered by Robert Brown in 1831 (Wilson, 1925), there still remains no general agreement as regards its real physical constitution. For example, one reads in Wilson's "The Cell in Development and Heredity," that the resting "vesicular nucleus, as seen in sections, usually shows four distinct components, namely: an enclosing wall or membrane; a nuclear framework usually described as a network or reticulum, though by some observers regarded as an alveolar structure; the nuclear sap, enchylema, or ground substance which occupies the interstices of the framework; and one or more nucleoli, massive and usually rounded bodies suspended in the framework." On the other hand, some investigators express the opinion summarized by Gray (1931) in his " Textbook of Experimental Cytology " that " Since all nuclei exhibit a visible granular or fibrillar structure after coagulative fixation, it is generally supposed that the structures seen in preserved preparations or in moribund nuclei are to be regarded as purely artificial products of coagulation, which cannot be correlated with the fundamental structure of the living nucleus. This view, developed many years ago by Hardy (1899), is now accepted by the majority of animal cytologists (Champy, 1913; Policard, 1922; Lewis and Lewis, 1924; Chambers and Rényi, 1925; Schitz, 1925; Burrows, 1927)."

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[2] Eggs of *Ascaris suum* will recover and divide after ultracentrifuging at 400,000 times gravity for an hour; we have recently centrifuged *Ascaris* eggs at 150,000 times gravity for 10 days during which time they divided. (In press, Science, 1936.)

It will be clear to those who analyze the literature on the physical nature of the nucleus that those who support the former view are mainly morphological cytologists, who have worked chiefly with fixed materials (however, see Bělař, 1929; Cleveland, 1935) and that those who support the latter view are chiefly experimental cytologists, who have worked mainly with surviving cells. In other words, the question resolves itself into whether or not the chromatin, " reticulum," nucleolus and nuclear " sap " seen in fixed preparations of the " resting " nucleus, correspond to the condition in the living nucleus to the same degree as do the chromosomes and other elements of the dividing nucleus.

The results reported in this paper seem to bear upon these questions, insofar as we have demonstrated that after centrifuging the living nucleus and immediately fixing and staining it, all the structures mentioned above are present, but displaced and stratified in the order of their relative specific gravity. This can only be interpreted to mean that the nucleus is not a homogeneous body, although it often appears so in the living material. It means, we think, that it is composed of materials of different relative density, yet of similar index of refraction, which usually prevents its elements from being seen in the living nucleus. Moreover, it is significant that these displaced nuclear elements appear morphologically similar in structure to those of the uncentrifuged fixed nucleus. Furthermore, the chromatin and " reticulum " materials in the centrifuged and subsequently fixed preparations take up the same relative position in the nucleus as does the prophase chromatin and chromosomes and hence must be of a similar specific gravity. Therefore, these results as well as the observations of the majority of cytologists have convinced us that well-fixed preparations of the " resting " nucleus probably give as reliable a picture of the condition during life as do fixed preparations of the prophase nuclei and chromosomes.

We, of course, realize that the structure of the "resting" nucleus no doubt varies greatly in different types of cells. For example, the chromatin in the nuclei of certain insect ova (Gardiner, 1935) is very scarce (diffuse?) as determined by the Feulgen method. In contrast to this, however, the nuclei of the salivary glands of many dipterous larvæ give a very marked reaction as of very orderly arranged segments of chromatin on an achromatic framework ("reticulum"). It would seem almost incredible that in the nuclei of the salivary glands of, for instance, *Chironomus* larvæ (King and Beams, 1935), all the material of the nuclei, except for the nucleolus, is evenly dispersed in a homogenous mass throughout the nucleus and subsequently clumped by fixation into long chromosomes, the identity of which may be recognized from cell to cell. In this connection, Doyle and Metz (1935) have recently shown, in the living nucleus of the salivary glands of *Sciara* larvæ, that no chromosomes or chromatic segments, so characteristically seen in aceto-carmine preparations, are to be observed. They nevertheless conclude from their studies that the chromosomes and chromatin segments are always present in the living nucleus, but that their refractive index is such that they are not optically differentiated.

Němec (1929) concludes from his work on centrifuged plant nuclei that a definite "reticulum" is present which is attached to the nuclear membrane. From our studies we cannot be sure just what constitutes the so-called "reticulum"; that is, whether it represents an achromatic framework in the nucleus impregnated with chromatin or whether it consists entirely of chromatin material. We are inclined to think that the "reticulum" in preserved cells has not been precipitated by fixation from a homogenous mass, but that it corresponds to a special material similarly distributed in the living "resting" nucleus.

When an inquiry is directed toward the structure and function of the mitotic figure, particularly the centrioles and spindle, one encounters an enormous amount of literature containing a most extraordinary group of conflicting contentions (see Wilson, 1925; Sharp, 1934; Schrader, 1934 and Bleier, 1930, for a review of the literature). It is beyond the scope of this paper to deal generally with a discussion of the mechanism of mitosis, but our results do seem to bear upon certain points of the structure of the mitotic apparatus which will be discussed below.

The reality of centrioles in living cells has recently been seriously questioned by Fry (1929). However, the centrioles in our material still possess their typical characteristics after being moved through the cytoplasm to the centripetal pole of the cell where they are in some cases detached and rest freely in the cytoplasm. This we believe is good evidence for their existence in the living cell. Furthermore, the fact that they have been so clearly demonstrated in the living cell by Cleveland (1935) and their genetic continuity so carefully followed by Heuttner (1933) leaves little doubt that they are actually present.

As regards the spindle, one group of investigators of which Chambers (1924) and Lewis and Lewis (1924) are typical, holds that the mitotic spindle is optically homogeneous, and the appearance of fibers in fixed preparations is an artifact, without any correspondence to structures in the living cell. However, in contrast to this view is the one derived by Cleveland (1935) from his work on living protozoan cells. Here "there is not the slightest doubt regarding the existence of the centrioles, the formation of the achromatic figure from the centrioles, the fibrillar nature of the achromatic figure, and the rôle of the achromatic figure in nuclear division." Our work points to the fact that the spindle and centrioles in chick cells are, in general, distinctly lighter than the chromosomes and for the most part lighter than the surrounding cytoplasm, as is evident by their frequent movement to the centripetal pole of the cell. The fact that the spindle is capable of considerable bending and distortion, without becoming detached or dissolved from the chromosomes or centrioles, argues that it is a body of considerable form and structure rather than an artifact. However, from our work we cannot definitely say that fibers are actually present in the living spindle, but certainly the structure of the spindle is capable of considerable tension and plasticity; it may be partially split and still possess its typical appearance in fixed preparations. These observations, we believe, support those of Cleveland (1935), who was able to establish by pulling the centrioles that the spindle fibers are real and definitely attached to the chromosomes. Recently Schrader (1934) has centrifuged dividing cells of crustaceans, molluscans and insects, with much less force than used by us. He states that "metaphases so treated continue their mitotic activities and it is concluded that the half-spindle components as seen in fixed preparations are not coagulation artifacts but have a morphological basis in the living spindle." Furthermore Wyckoff (1934), who attempted to photograph (with ultraviolet light) the living spindle, says "you surely see something; but whether it is the individual fibers or the result of a state of strain in the viscous protoplasm, I don't know." Lewis (1934) has recently reported that when the metaphase spindle of chick cells in tissue culture is exposed to a hypotonic medium, the fibers disappear, the chromosomes are dispersed from the equatorial plate and the progress of division stopped. However, if the hypotonic medium be replaced in time by an isotonic medium, a new spindle forms, the chromosomes collect on the equatorial plate, and division follows. If the cell is fixed and stained after the action of the hypotonic medium but before the addition of the isotonic medium, no spindle fibers are to be found, although the centrospheres seem not to have disappeared. These observations indicate definitely that the spindle is essential for division in chick cells, and that it is capable of being dissolved and reformed while the cell is in the metaphase state of division. In any case, whatever the structure of the spindle may be, we are in agreement with Seifriz (1929) " that it makes little difference whether we regard the spindle fibers as threads, rows of granules or lines of force. If fixation causes previously existing lines of force to appear as fibers this is sufficient, for, after all what are lines of force if not the linear orientation of particles, whether electrons or protoplasmic granules."

CONCLUSIONS

1. Chick embryonic cells, both " resting " and in stages of division, have been centrifuged at approximately 150,000 times gravity for 10 minutes.
2. The elements of the " resting " nucleus were stratified in the order of their decreasing specific gravity as follows: (*a*) nucleoli; (*b*) chromatin and " reticulum "; (*c*) nuclear " sap."
3. Evidence is presented to support the view that fixed preparations of the " resting " nucleus give as characteristic a representation of the elements during life as do fixed preparations of prophase nuclei and metaphase chromosomes.
4. The metaphase spindle has been distorted, its chromosomes displaced, its fibers separated, and, in some cases, it has been completely disintegrated by centrifuging.
5. These results support the claim that spindle fibers and centrioles as seen in fixed preparations have a morphological basis in the living spindle.

LITERATURE CITED

BEAMS, H. W., AND R. L. KING, 1934*a*. The effects of ultracentrifuging upon the Golgi apparatus in the uterine gland cells. *Anat. Rec.*, **59**: 363.

BEAMS, H. W., AND R. L. KING, 1934*b*. Effect of ultracentrifuging on the mitochondria of the hepatic cells of the rat. *Anat. Rec.*, **59**: 395.

BEAMS, H. W., AND R. L. KING, 1935. The effects of ultracentrifuging the spinal ganglion cells of the rat, with special reference to Nissl bodies. *Jour. Comp. Neur.*, **61**: 175.

BĚLAŘ, K., 1929. Beiträge zur Kausalanalyse der Mitose. II. Untersuchungen an den Spermatocyten von Chorthippus (Stenobothrus) lineatus Panz. *Arch. f. Entw. Mech.*, **118**: 359.

BLEIER, H., 1930. Untersuchungen über das Verhalten der verschiedenen Kernkomponenten bei der Reduktionsteilung von Bastarden. *La Cellule,* **40**: 83.

CHAMBERS, R., 1924. The Physical Structure of Protoplasm as Determined by Micro-dissection and Injection. General Cytology (Edited by Cowdry), p. 272.

CLEVELAND, L. R., 1935. The centriole and its rôle in mitosis as seen in living cells. *Science,* **81**: 598.

DOYLE, W. L., AND C. W. METZ, 1935. Structure of the chromosomes in the salivary gland cells in Sciara (Diptera). *Biol. Bull.,* **69**: 126.

FRY, H. J., 1929. The so-called central bodies in Cerebratulus eggs, etc. *Biol. Bull.,* **57**: 131.

GARDINER, M., 1935. The origin and nature of the nucleolus. *Quart. Jour. Micr. Sci.,* **77**: 523.

GRAY, J., 1927. The mechanism of cell-division. IV. The effect of gravity on the eggs of Echinus. *Brit. Jour. Exper. Biol.,* **5**: 102.

GRAY, J., 1931. A Textbook of Experimental Cytology. Cambridge.

HARVEY, E. B., 1935. The mitotic figure and cleavage plane in the egg of Parechinus microtuberculatus, as influenced by centrifugal force. *Biol. Bull.,* **69**: 287.

HEIDENHAIN, M., 1907. Plasma und Zelle. (Jena.)

HERRICK, F. H., 1895. Movements of the nucleolus through the action of gravity. *Anat. Anz.,* **10**: 337.

HUETTNER, A. F., 1933. Continuity of the centrioles in Drosophila melanogaster. *Zeitschr. f. Zellforsch. u. Mikr. Anat.,* **19**: 119.

KING, R. L., AND H. W. BEAMS, 1934. Somatic synapsis in Chironomus, with special reference to the individuality of the chromosomes. *Jour. Morph.,* **56**: 577.

KING, R. L., AND H. W. BEAMS, 1934. Effect of ultracentrifuging on Paramecium. *Proc. Soc. Exper. Biol. and Med.,* **31**: 707.

LEWIS, M. R., 1934. Reversible solation of the mitotic spindle of living chick embryo cells studied in vitro. *Arch. exp. Zellforschg.,* **16**: 159.

LEWIS, W. H., AND M. R. LEWIS, 1924. The behaviour of cells in tissue cultures. General Cytology (Edited by Cowdry).

LILLIE, F. R., 1909. Karyokinetic figures of centrifuged eggs; an experimental test of the center of force hypothesis. *Biol. Bull.,* **17**: 101.

LUYET, B. J., 1935. Behavior of the spindle fibers in centrifuged cells. *Proc. Soc. Exper. Biol. and Med.,* **33**: 163.

MORGAN, T. H., 1927. Experimental Embryology. Columbia University Press.

NĚMEC, B., 1928. Über die Beschaffenheit der achromatischen Teilungsfigur. *Arch. exp. Zellforschg.,* **5**: 77.

NĚMEC, B., 1929. Über struktur und aggregatzustand des Zellkernes. *Protoplasma,* **7**: 423.

SCHRADER, F., 1934. On the reality of spindle fibers. *Biol. Bull.,* **67**: 519.

SEIFRIZ, W., 1929. The structure of protoplasm. *Biol. Rev.,* **4**: 76.

SHARP, L. W., 1934. Introduction to Cytology. McGraw-Hill.

SPOONER, G. B., 1911. Embryological studies with the centrifuge. *Jour. Exper. Zoöl.,* **10**: 23.

WILSON, E. B., 1925. The Cell in Development and Heredity. Macmillan, N. Y.

WYCKOFF, W. G., 1934. Ultraviolet microscopy as a means of studying cell structure. *Cold Spring Harbor Symposia on Quantitative Biology,* **2**: 39.

THE EFFECTS OF HEAVY WATER UPON THE FISSION RATE AND THE LIFE CYCLE OF THE CILIATE, UROLEPTUS MOBILIS

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Since the discovery of the heavy isotope of hydrogen, a great number of "biological" experiments have been conducted with a variety of concentrations and with a diversity of complex phenomena such as growth, longevity, enzymatic reactions, oxygen consumption, luminescence, and permeability relations. For reviews of the current literature Barnes and Jahn (1934) or Fox (1934) may be referred to. In general, the work with low concentrations of deuterium shows a diversity of results; contradictions frequently occur as to the effects upon the same biological processes. A depressing effect has not usually been found. On the other hand, indications of stimulation or of the absence of effects exist.

In the work with Protozoa, a stimulating effect in respect to population numbers was reported with *Euglena gracilis* in D_2O of 1.000061 density by Barnes (1934). This would indicate that some life processes are speeded up in this weak concentration; Barnes and Larson (1934) also hold that the life span of primitive cells such as *Spirogyra* is increased by dilute solutions (e.g. 0.06 per cent) of deuterium. It appeared to have little or no effect upon the rate of cell division or upon the rate of elongation of the cells. The autodigestive processes of the planarian, *Phagocata gracilis,* during starvation were considerably slowed down in 0.06 per cent. With a concentration of 0.13 per cent to 0.47 per cent D_2O, the effects were "progressively obscured" due to the increase of bacteria and molds. Clearer results probably could have been obtained by a frequent change of the solutions.

Harvey (1934) as well as Taylor, Swingle, Eyring, and Frost (1933) reported no toxic effects with low concentration upon *Paramecium caudatum;* however, no actual account of fission rates or length of life was given.

Barnes (1935) also stated that "workers who claim that dilute heavy water has no influence on living organisms have not allowed sufficient time for the appearance of recognizable effects." It was with this very purpose that an analysis of the fission rate of the ciliate protozoan, *Uroleptus mobilis,* during its complete life cycle was made. Since fission rate is one of the indications of the state of vitality of this protozoan, it was proposed to find whether a dilute concentration of deuterium oxide would alter this vitality over a long period of time, and if it would produce a stimulating effect such as has been reported upon the flagellate, *Euglena,* by Barnes. Secondly, it was proposed to note any changes in the total longevity of the culture. This type of isolation work involves no obscuring of effects by molds or bacteria since each animal is placed daily into fairly constant, fresh medium.

With intermediate concentrations, Taylor, Swingle, Eyring, and Frost (1933) reported that *Paramecium caudatum* may survive for about 72 hours in 60 per cent to 70 per cent D_2O, although many die after 48 hours. A concentration of about 50 per cent was selected to test the effects upon the fission rate of *Uroleptus mobilis* and its length of survival. The contractile vacuole rate was also observed as an additional method of noting any changes upon direct immersion into 50 per cent D_2O. The results will be compared below with those obtained by Barnes and Gaw (1935) for the immersion of *Paramecium caudatum* into 30 per cent D_2O.

The work with high concentrations has yielded relatively consistent results, namely that the protoplasm of all organisms cannot survive for more than short periods. Several Protozoa have been tested. Harvey (1934) found that *Amœba dubia,* as well as an unnamed species, was killed in 6 to 20 hours, and that although *Euglena proxima* exhibited avoiding reactions, then rounded up and became immobile soon after immersion in 90 per cent D_2O (a certain proportion remaining mobile, however, for three days), activity can be regained after five days by a return to normal water. With *Euglena gracilis,* recovery was possible after nine days in the D_2O, thus indicating that the animals were not "irreversibly injured." However, whether the treated animals would show delayed effects in their subsequent life history was not tested. Taylor, Swingle, Eyring, and Frost (1933) found a similar decrease in motility for *Euglena viridis* in 92 per cent D_2O, although recovery tests were not made. *Paramecium caudatum* swam slowly upon immersion into 97 per cent D_2O; in two hours the contractile vacuole enlarged with a cessation of contraction; blisters formed and it died within less than 24 hours (Harvey). Taylor, Swingle, Eyring, and Frost state that more time was required to kill *Paramecium caudatum* than frogs, fish, or flatworms, and they note that "*Paramecium* shows more resistance to heavy water than highly organized animals studied." If *Uroleptus mobilis* had been selected by chance instead of *Paramecium,* it would have been found that no more time for disintegration was needed, since this ciliate usually collapses at once. This scattered work points to the fact that until a systematic immersion of various types of Protozoa is made to answer such questions as the correlation of the kinds of pellicles with the degree of resistance or whether animals in the encysted state may be more resistant to high concentrations, no general comparisons can be accurately made.

I wish to express my appreciation to Dr. Gary N. Calkins for the suggestion of this work and for his helpful advice, to Dr. James Curry for the making of the dilutions, to Dr. T. M. Sonneborn for his criticisms, and to Dr. H. S. Jennings for permission to complete the work at the Johns Hopkins University. The deuterium oxide was supplied by the Chemistry Department of Columbia University upon a grant from the Rockefeller Foundation.

Material and Methods

Cultures in 0.44 Per Cent Deuterium Oxide

The descendants from each member of two pairs of conjugating *Uroleptus mobilis* which were taken from stock mass cultures were selected for study. From each single ex-conjugant one series of descendants was carried in isolation cultures throughout the life cycle; these series will be designated as *A, B, C,* and *D* respectively. Series *A* and *B* are derived from the members of one of these conjugating pairs; *C* and *D* from the other. In each series there were six isolation lines, each line being one continuous sequence of descendants.

The medium for the normal control lines was made in the same way as that of Calkins for *Uroleptus* (1919); 50 mg. of timothy hay were boiled for 5 to 10 minutes in 50 cc. of Great Bear Spring water to which a few grains of white flour were also added. A second type of medium was prepared in the same manner, except that redistilled water was used instead of the usual spring water. For the medium of 0.46 per cent D_2O, a modification in preparation was made due to the sufficient but limited supply of the material. In order to produce a corresponding concentration of daily food supply in the 0.46 per cent D_2O, 50 cc. of distilled water with the same proportions of hay (i.e. 50 mg.) and flour as above were boiled to 10 cc. The same amount of hay was again added and the liquid was further boiled to 1 cc. and allowed to remain for 24 hours. The D_2O medium was then made up in the proportions of one drop of concentrated medium to 24 drops of 0.46 per cent D_2O; the concentration of D_2O was thus lowered to about 0.44 per cent D_2O. The 0.46 per cent D_2O came from the same lot as that used by Curry, Pratt, and Trelease (1935) (for preparation of which, see same).

All lines of each series were run for the first four ten-day periods in normal medium. During Period 5 in Series *B, C,* and *D,* and Period 6 in Series *A,* two lines of each series were transferred into the redistilled water medium and two lines into the 0.44 per cent D_2O by gradual changes extending over six days. For protection against evaporation and change of concentration of cultures, the depression dishes were fixed with vaseline covers. The cultures were continued in the same type of medium until the end. When one line died, a substitution of an animal from another line in the same type of medium was made.

The average fission rates for ten-day periods were calculated after Calkins (1919). If a line was ended by death of an animal during the ten-day period, it was not used in the calculation. If death occurred in all lines which were in the same medium at various times during a ten-day period, the average was made by averaging the fission rates of the preceding and succeeding periods (Calkins). Since the animals were under unfavorable conditions of transportation during Period 8, the values were also calculated in this way. For the first four periods, the six lines of one series were averaged together.

During Periods 1 to 4, the animals remained at room temperature which was around 24° C. An extreme temperature variation killed some of the animals in Period 4; the cultures were thereafter kept at a temperature of 24° to 28° C. Although this temperature variation doubtless accounts for some of the variation in the fission rate from period to period, it introduces no error into comparisons made synchronously, since all cultures were exposed to the same temperature at any one time.

Cultures in 48.1 Per Cent Deuterium Oxide

The descendants from one ex-conjugant of a conjugating pair which was selected from a stock culture of *Uroleptus mobilis* were carried in six lines for the controls. All lines which were started in the 50 per cent D_2O medium or in the special control water were derivatives from the control lines; in all cases, six lines of each type were carried. The normal and heavy water media were made up in the same way as those in the former experiment except that 50 per cent D_2O was used instead of 0.46 per cent D_2O.

The 50 per cent D_2O was prepared by the dilution of purified 95 per cent D_2O. For a part of the experiment, the animals were subjected to a special type of control water which was put through the same process as that used for the preparation of the deuterium of high concentrations, except that it had a concentration of deuterium which corresponded to ordinary distilled water. After dilution with the necessary food solution, the 50 per cent water was about 48.1 per cent. From Period 10 on, distilled water was substituted for the spring water in the controls.

From Period 1 to 6, the temperature ranged from 24° C. to 28° C. During Periods 6 through 8, the animals were transported several times and remained at room temperature. After Period 8, they were kept at 22° to 27° C.

In order to test the abundance of the bacteriological food supply in the 48.1 per cent D_2O, four samples of the medium after 24 and 48 hours were plated upon nutrient agar which was made up with the 50 per cent D_2O, and were compared with four samples from the control lines made upon normal agar upon the same day during Period 25 and at the same temperature. As much care as possible was taken to plate approximately the same amounts of fluid by following one type of procedure, although this type of quantitative work cannot be accurate even at its best.

In twelve cases, the contractile vacuole rate of *Uroleptus mobilis* was observed before, after, and during immersion into 48.1 per cent D_2O. The specimens came from the isolation lines of the group of animals which was used as controls in testing the effects of 48.1 per cent D_2O upon the fission rate; they were taken at random, but all came from Period 19 in which the average fission rate was 13.1. Before observing, the animals were placed in several drops of fresh, normal medium for one to two hours. The animals were placed in one drop of the 48.1 per cent D_2O medium in a depression slide covered with a vaselined coverglass for observation. The D_2O medium was made up with nutrient in the same manner as in testing the fission rates. Upon immersion of the animal, the fluid was carefully mixed with the pipette. Upon removal, the animal was briefly washed through three or four drops of the normal medium, before observing. For any continued observation the animal was transferred to fresh medium every two hours. The figures used represent the averages of the number of seconds between successive contractions of the vacuole, based upon about ten consecutive measurements recorded by the use of two stop watches.

Results

Low Concentration

In mass cultures from all three types of media, i.e., the spring water, redistilled water, and the 0.44 per cent D_2O medium, normal and similar amounts of conjugants as well as encystments were obtained during the life cycle. Thus 0.44 per cent D_2O has no striking effect, as revealed by this general method, upon the ability of animals to conjugate or encyst.

In considering the effect of the low concentration of 0.44 per cent D_2O upon a measurable function such as the fission rate of the ciliate, *Uroleptus mobilis,* no changes beyond the normal variation occurred, not only during a short time such as ten to twenty days, but also during a prolonged contact for at least ninety days with this low concentration of D_2O. No accumulative or delayed effects were found, as may be seen from a comparison of the graphs for the four series in Fig. 1. For the most part, the 0.44 per cent D_2O lines followed those in redistilled water more closely than those in the normal control medium. Except for short divergences in these two media such as in Series *A,* Period 6; Series *B,* Periods 10 and 14; Series *C,* Period 12; and Series *D,* Periods 10 and 12; the rates were almost identical throughout the seventy or more days. In all media the appearance of the animals was normal.

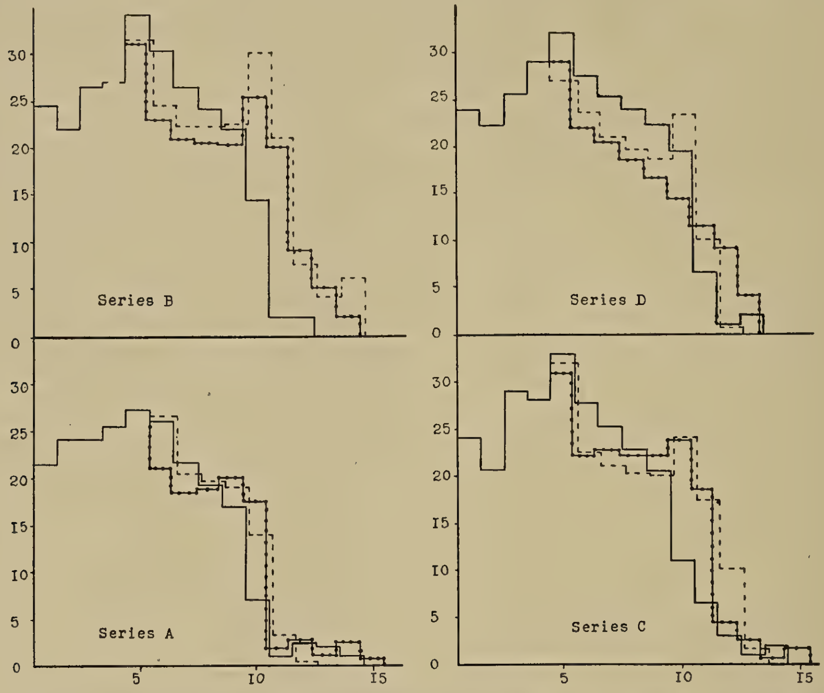

FIG. 1. Average number of fissions for four series: *A, B, C,* and *D,* during the life cycle in media made with spring water (——), redistilled water (– – –), and 0.44 per cent D_2O (–•–•–). Ten-day periods (abscissæ) are plotted against the average number of fissions for ten days (ordinates).

Furthermore, in considering the total number of generations, it was found that the average number for the animals in 0.44 per cent D_2O cultures was intermediate in three series (*A*, *B*, and *C*). In Series *D*, the average of 233.5 generations in 0.44 per cent D_2O was slightly less than that of 242.5 generations for the redistilled water cultures or 254.5 for the normal controls. These differences in all cases lie within the limits of normal variation. In no series is there even a slight indication that the total longevity is prolonged or cut short by the contact of the animals with this low concentration of D_2O.

The percentage of animals surviving in the 0.44 per cent D_2O for seven periods after immersion was of the same magnitude as in the other two media for the same length of time. During this time, not more than two deaths occurred in any one line; there was only one death in all four series during the transitional period in the 0.44 per cent D_2O lines.

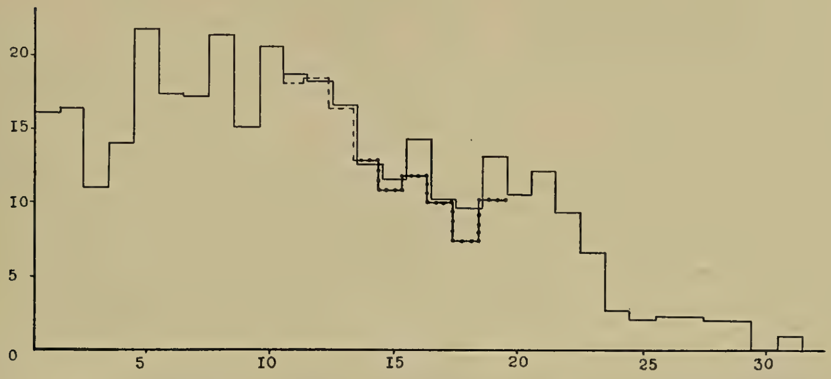

FIG. 2. Comparison of fission rate of Group *A* (——) in normal medium, and Group *B* (– – –) in special control water put through the same process as the 48.1 per cent D_2O. Return to normal medium –·–·–. Abscissa = ten-day periods. Ordinate = mean number of fissions in ten days.

Intermediate Concentration

Conjugation and encystment tests made on animals which were living in normal spring water or in special control water usually yielded many viable conjugants and cysts. In the greater proportion of cases in the 48.1 per cent D_2O, conjugation was not obtained since the conditions of the medium became toxic before enough animals could be obtained by their slow division to produce conjugating conditions. In the few cases where enough animals were obtained, conjugation always occurred in good proportions but the ex-conjugants never survived. In two to three days, after conjugation in the culture, the ex-conjugants became small, either subsequently disappearing or forming what appeared to be cysts.

Attempts at ex-cystment were unsuccessful. Similar results for the ex-conjugants from 128 conjugating pairs which were isolated in fresh D_2O medium were obtained. These also could not be revived in 48.1 per cent D_2O and disappeared after a time. Twenty-four conjugating animals were placed in normal medium; 3 of the ex-conjugants died within ten days, but the other 21 survived. Their subsequent fission rates, however, were not tested.

The normal control animals will be designated as Group *A*; derivatives from them, as Group *B*, as shown in Fig. 2. The former group survived for 31 ten-day periods and its fission rate was practically the same as that of Group *B*. This similarity existed both in Periods 11 to 13, when Group *B* was carried in special control water; and during the following six periods, when Group *B* was carried in normal medium. This shows that any changes found in the 48.1 per cent D_2O medium are not due to the presence of injurious agents, since this special control water was carried through the same process as the 95 per cent D_2O from which the 50 per cent D_2O was diluted.

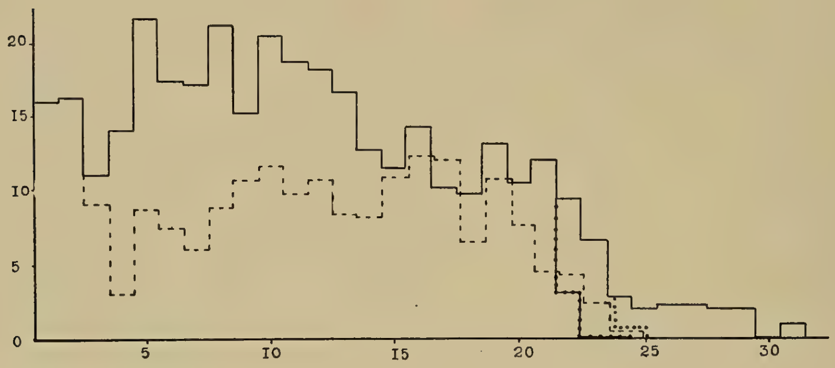

FIG. 3. Effects of 48.1 per cent D_2O on fission rate and length of life cycle of animals immersed in it at various ages. Group *A* (——), in spring water; Group *C* (– – –), *D* (–·–), and *E* (· · ·), in 48.1 per cent D_2O. Group *C* introduced into D_2O when mature, Groups *D* and *E* introduced into D_2O when old. Abscissa = ten-day periods. Ordinate = mean number of fissions in ten days.

At the end of Period 2, when the animals were in the forty-fifth to forty-eighth generations, derivatives (Group *C*) from each of the control lines were placed in 48.1 per cent D_2O, and allowed to remain until they died (Fig. 3). During the first fifty days (i.e., Periods 3 to 8) the fission rate decreased to about half that of the normal animals so as to approximate that of old animals in a declining period such as No. 22 or 23. After a slight rise in Periods 8 to 10, the rate continued near the new level for about ninety days to Period 20. A decrease set in which ended in the death of the animals in about fifty more days. The slight increase in the rate may be interpreted as a delayed adjustment of the animals to the new environment, but due to the chemical or physical effects of the D_2O, the adjustment or return to the normal level was impossible. From Periods 15 to 20, the rates of both the control animals and those in 48.1 per cent D_2O were at the same level because the former were in the early stages of their decline.

It is also seen from Fig. 3 that although 48.1 per cent D_2O depressed the fission rate greatly during " maturity " (i.e., that time during the life cycle in which the animals are dividing at a high rate), the animals showed " aging " (the first signs of a general decline in the fission rate) at about the same time as the controls. However, " senescence " (period of very low fission rate which is followed by death) was cut much shorter.

Not only did the animals which were placed in 48.1 per cent D_2O during a period of high vitality divide more slowly but they also did not live as long. The animals began to die off in Periods 23 to 24, with an end to all lines in Period 25; while the controls did not start to die off until Periods 27 and 28, and ended in Period 31. Consequently, since the fission rate was lower in 48.1 per cent D_2O and since the length of life was shorter, the total number of generations was less, an average of 220.6 generations as compared to one of 366.1 for those in normal medium.

That these changes, in deuterium of this concentration, were not due to a continued insufficient diet can be assumed from the results of bacterial checks on the medium. In each case of the samples taken after 24 and 48 hours from the normal and heavy water cultures, the number of bacterial colonies upon nutrient agar was approximately the same. Also the same types of colonies in the same proportions appeared. Although no further examination of exact ratios was made, this roughly indicated that the bacterial food supply was at least ample in quantity and quality.

When normal old animals (Group *D*) in the 328th to 346th generations at the end of Period 21 were placed in 48.1 per cent D_2O, the fission rate fell to 3.0 and soon to zero (Fig. 3). By Period 25 all lines were dead, thus living only for a short time. Although these animals were dividing at a rate of 12.0 fissions per ten days just before immersion as compared with that of 16.3 fissions per ten days for the first

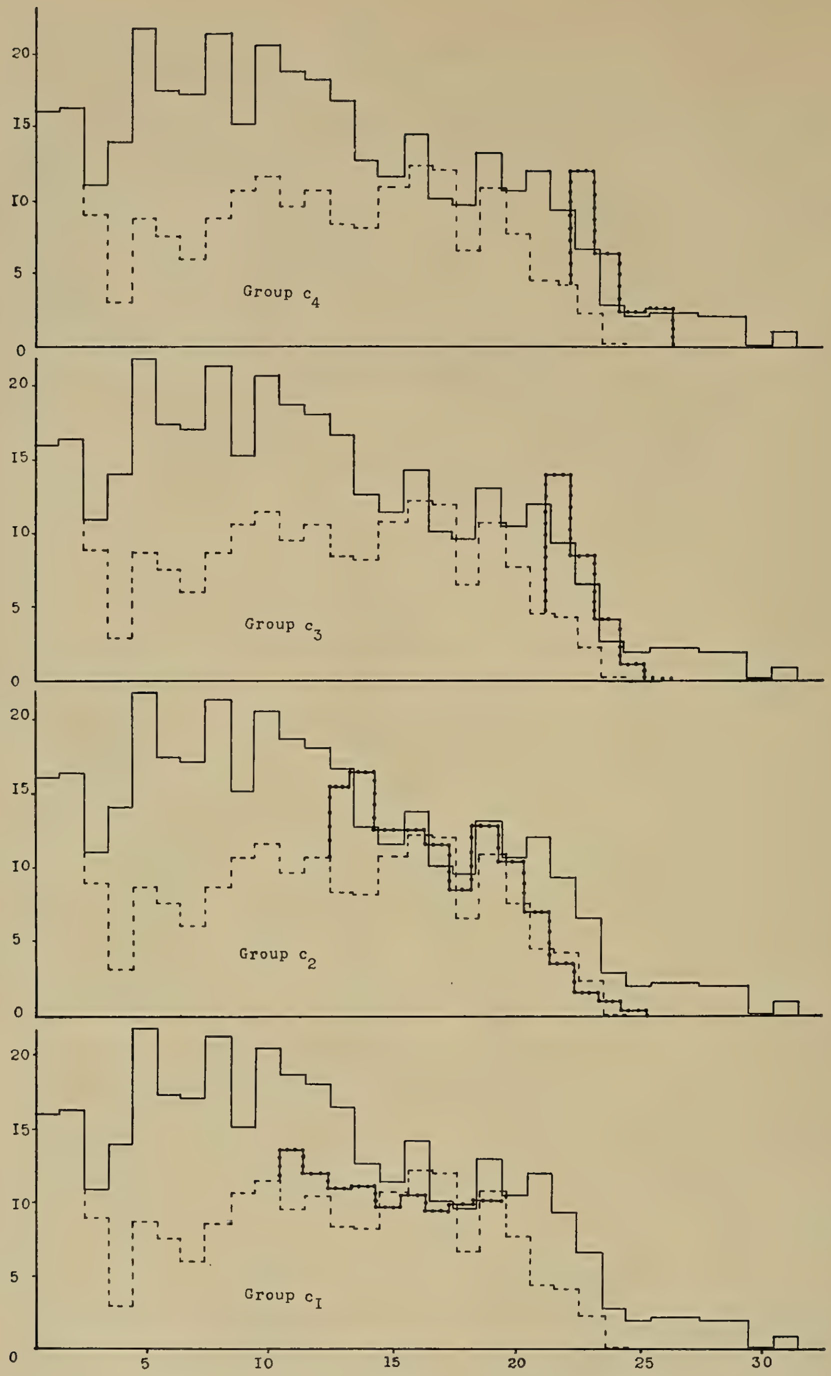

FIG. 4. Fission rate and total longevity of mature animals after release from long exposure to 48.1 per cent D_2O for various lengths of time. Normal controls, Group *A* (——); Group *C* in 48.1 per cent D_2O (---); Group c_1, c_2, c_3, and c_4 after return to normal medium (–·–). Abscissæ = ten-day periods. Ordinates = mean number of fissions in ten days.

group which was immersed after Period 2, it does not seem probable that this difference would account for the short survival time of Group *D*, but that the latter is due to the age of the animals upon immersion.

Similarly, when a third group (Group *E*) was placed in 48.1 per cent D_2O at the end of Period 23 (Fig. 3), the survival time was less than one period. The longest length of life of any individual was thirteen days while the average was five days for this group. Again after Period 27 when the normal group was very old, similar results were obtained.

Derivatives from the animals in 48.1 per cent D_2O were placed back into normal medium at five points, i.e., at the beginning of Periods 11, 13, 22, and 23 (Groups c_1, c_2, c_3, c_4, respectively). In each case the fission rate rose during the first period of this return (Fig. 4); except for Group c_2 it did not closely approximate the normal rate at the same time. In Groups c_3 and c_4 the rate rose to a level which was above that of the normal controls for the same period. Those in the first lot to be removed (c_1) were placed in the special control water which was later substituted by the normal medium after Period 13. The other groups were placed directly in normal medium. Since the first group was discontinued after Period 19, no further statements can be made concerning it. None of these released groups which were continued to the end of the life cycle lived as long as the normal control animals. Some were also removed at the end of Period 25; they died within a few days with no divisions, thus following the same course as the animals in the 48.1 per cent D_2O at that time. Since all of these groups died before the animals in the normal medium, they must have been slightly weakened by the long contact with the D_2O.

On the other hand, when derivatives (Group d_1) were removed at the end of Period 24 from the group of old animals which had been placed into 48.1 per cent D_2O from normal medium after Period 21 (Group *D*), they lived as long as the normal controls (Fig. 5). The release was accompanied by a stimulation which caused the fission rate to rise to 7.7 during the first ten days while the controls (Group *A*) were dividing at a rate of only 2.1. However, this high rate declined in the next sixty days so that the animals died at the same time as the normal controls. In spite of their age, the relatively short time of thirty days in D_2O had no permanent harmful effects, although the rate was strongly suppressed while in this medium.

For a secondary indication that the intermediate concentration changes life processes, a few measurements of the contractile vacuole rate were made. In transferring a normal *Uroleptus mobilis* to 48.1

per cent D_2O from the normal medium, there was an immediate decrease in the rate (i.e., within 15 minutes or less). Out of the twelve cases examined, there was one exception in which no regulation occurred and the animal suddenly disintegrated within two hours while still in the heavy water. Whether or not this lack of regulation was the cause of the death cannot be said. The general decrease was greater than any sudden fluctuations which occur in the normal rate. For six animals measured over a period of 4 to 5 hours with observations every 20 minutes in the normal medium, the greatest change was 3.9 seconds in 20 minutes; while upon transfer into 48.1 per cent D_2O, the decrease varies in extent from 6.1 seconds to 21 seconds for different animals. The majority fell between 7 and 15 seconds, although an insufficient number were tested for averages. Consecutive immersions were not tried. Immediately upon immersion, in some cases, the contraction of the vacuole was very irregular, although within half an hour it became fairly constant. The degree of decrease did not depend upon the initial rate. Therefore each animal seemed to regulate itself according to its individual state at the time of immersion. The obtained variation in the decrease occurs within a temperature range of 2° C.

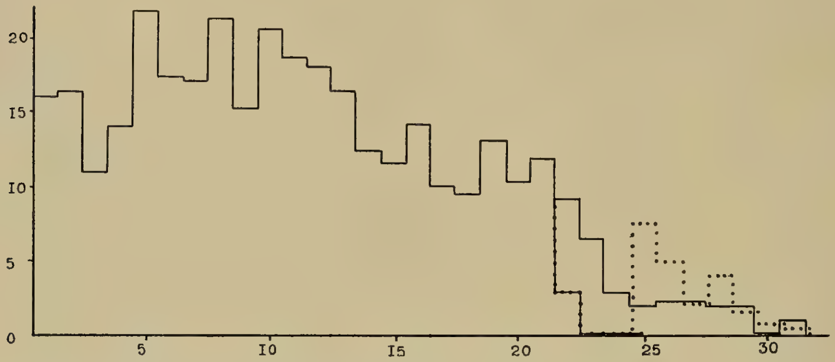

FIG. 5. Fission rate and total longevity of old animals after release from a short exposure to 48.1 per cent D_2O. Normal medium, Group *A* (——). Group *D*, animals put into D_2O when old (–·–·–). Group d_1, animals returned to normal medium after 30 days in D_2O (···). Abscissa = ten-day periods. Ordinate = mean number of fissions in ten days.

It is very difficult to assign one value to the rate while the animal is in any effective concentration of deuterium for a short time because the rate may fluctuate. The initial decrease may not be lasting or constant. In considering four animals which were kept in the D_2O from four to six hours, one of these animals regained and increased above its normal initial rate in less than three hours after immersion, although its original decrease upon immersion was as great as that of the other three animals which continued to show a low rate in the D_2O until their release. A more extensive experiment would probably show more of this type. Also in the seven other cases examined over a much shorter period of time, there were two which gained the approximate original level in less than one hour and one which showed an increase in the rate just before it was released. Here also the initial change was as great as in the four other cases whose rates did not increase while in the D_2O. Whether any of these animals would have reached the original level after four to six hours cannot be told. There is also the possibility that these fluctuations are only preliminary and that over a longer period of time such as 24 hours a marked difference in the 48.1 per cent D_2O might show.

TABLE I

Differences in contractile vacuole rate between animals living in normal water (Group *A*) (Group c_1) and those in 48.1 per cent D_2O (Group *C*).

	Average contractile vacuole rate	Number of animals observed
	seconds	
Normal controls.		
Group *A*	19.7	13
In 48.1 per cent D_2O.		
Group *C*	28.9	27
After release from 48.1 per cent D_2O.		
Group c_1	20.6	8

This possibility is further substantiated by data obtained from measuring the contractile vacuole rates in samples of animals taken from Group *A* (normal medium) and Group *C* (48.1 per cent D_2O) during Periods 9–15 inclusive, as well as from Group c_1 (normal medium after eighty days exposure to 48.1 per cent D_2O) in Periods 12–15 inclusive. Table I shows that there is a clear distinction between the average rate of animals in normal medium and those in the heavy water; the animals which had been returned to normal water for ten days (Group c_1) had already gained the normal rate.

Upon removal from exposure to D_2O for a short time there was a corresponding change in the contractile vacuole rate which varied in its magnitude for various animals. In nine cases out of the eleven which were tested (above), the change was as great or greater than the highest variability while the animal was still in the deuterium, so that removal as well as immersion may cause a real change in the rate. Variation was present here also. In most cases the change restored the rate to or near the original level in half an hour or less.

High Concentration

When normal vegetative animals were immersed in 98 per cent D_2O an immediate quick shrinkage occurred, resulting in the formation of spherical or somewhat truncated shapes with the cilia still beating. Usually in five seconds to one minute these balls disintegrated. In a few cases the animals survived in the shrunken state for more than two hours. One animal which became about half of its original size during four minutes in 98 per cent D_2O gradually regained its normal appearance after transferal to normal medium and lived for thirty hours. Usually there was no recovery from these short exposures. Even although most specimens die at once, some distinct variability is apparent in *Uroleptus mobilis.*

DISCUSSION

The facts indicate that neither the amount of conjugation or encystment in mass culture, nor its total longevity, nor the fission rate of *Uroleptus mobilis* during its life cycle are altered by a low concentration of deuterium and they are in harmony with indications from other work that low concentrations neither accelerate nor slow down certain life processes. On the other hand, these results are in contrast both to the stimulating effect upon the increase in population numbers of *Euglena gracilis* in mass cultures as reported by Barnes (1934), and to the increase of the life span of primitive cells such as *Spirogyra* (Barnes and Larson, 1934).

It is interesting to note that very young ex-conjugants from animals which have conjugated in the 48.1 per cent D_2O never survive if they are allowed to remain in this medium, but will live if the conjugants are transferred to normal hay infusion. Assuming from this latter fact that the conjugation process in the D_2O is regular, which should be checked as far as possible, the high susceptibility of conjugants and their young ex-conjugant derivatives is evident. Jollos has long maintained that conjugants as well as recent ex-conjugants are more sensitive to unfavorable conditions than animals during other stages. However, mature animals such as those which were placed in the 48.1 per cent D_2O twenty days after conjugation and subsequent fission to 45 to 48 generations in the normal medium, were more able to withstand the effects of this agent, although it is evident that their period of "senescence" was cut short. Furthermore, old animals, after 328 to

346 generations in the normal medium, have only a short survival in the deuterium. Although the discrepancy exists that the young animals conjugated in the D_2O and that the mature and old animals were derived from conjugation in normal hay infusion, these facts are in general accordance with the effects found by Gregory (1925, 1926) upon very young, mature and old *Urolepti* by continued treatment with beef extract or di-potassium phosphate, and by brief treatment with di-sodium phosphate, i.e., that both young and old series are immediately depressed while mature animals are stimulated in these media. Immature and old animals are not as able to adjust themselves to rapid changes in the environment. Thus these results not only show the actual effects obtained with an intermediate concentration of deuterium but also incidentally further substantiate the theory of Calkins (1926) that changes occur in the derived organization of *Uroleptus mobilis* throughout its life cycle.

That 48.1 per cent D_2O is clearly of a depressing nature is seen from the facts that both fission rate and total longevity are less in this medium. Also mature animals which have been in contact with this concentration for a long time, as from 90 to 230 days, are weakened. Although an immediate stimulation which may cause the fission rate to rise above that of the normal controls at the same time may occur upon release from heavy water, they do not live as long after they are returned to the normal medium as the control specimens which have never been exposed. On the other hand, even old animals are not weakened by shorter contacts as for 30 days, since they afterwards live as long as the controls; as above, the fission rate may even be temporarily stimulated upon release from heavy water to a rate above that of the normal controls. This clearly shows the necessity of carrying out work of this type with Protozoa over a long time in order to obtain the full results which may not be present at an earlier stage. This intermediate concentration, in contrast to the low concentration used, has a clearly depressing effect upon the protoplasm of *Uroleptus mobilis.*

The changes produced are not due to the lack of sufficient amounts or types of the bacterial food supply, nor are they due to any injurious impurity in the D_2O, since the fission rates of the animals in the special control water, which had been run through the same series of distillates as the D_2O, were equal to the normal rate.

With the few contractile vacuole rate measurements as a second way of testing the effects of an intermediate concentration, a slight decrease upon immersion is usually found. The great variation in the degree of decrease points to the fact that the evident attempts at regulation are a function of the particular individuality of the protoplasm at this time. Each animal regulates itself according to its state, which is unknown to the observer. The decrease in contraction may continue within the first four to six hours, or it may regain the original rate which the vacuole possessed before immersion. However, it might reach a steady decreased rate or a complete recovery in all cases after a longer period such as twenty-four hours. That the former condition is the more likely is seen from the fact that animals which had been running in isolation cultures in the 48.1 per cent D_2O for sixty days were contracting at an average rate of 28.9 seconds as compared with that of 19.7 seconds for the control lines in normal medium. From the work of Barnes and Gaw on *Paramecium caudatum,* a decrease was reported after twenty-four hours immersion in 30 per cent D_2O. Finally, upon removal from D_2O, there may be a corresponding increase in the contractile vacuole rate which usually brings it back to or near its original level in those animals whose rate has remained lowered in the heavy water solution.

In working with the contractile vacuole rate of *Paramecium caudatum,* Barnes and Gaw (1935) state that "in a race of *Paramecium caudatum* the contractile vacuole empties every 18.9 seconds in 30 per cent D_2O and 11.3 seconds in controls at 18.8° C." They apply the Arrhenius equation to their data and find that in 30 per cent D_2O, the graph representing the log K as a function of $1/T$ is an unbroken line throughout the entire temperature range which was used, and that E (or the energy of activation of the controlling catalyst) equals 22,000 calories. Moreover, they found that in normal medium, the rate varies with the temperature, and from the rates assigned specific values to E at these temperature ranges. Since E below 16° C. was similar to the E in 30 per cent D_2O, they concluded that deuterium has similar effects to those of low temperatures.

In the first place, the extremes of 18.9 seconds and 11.3 seconds for contraction are within the limits of normal variation for *Paramecium caudatum.* The normal extremes can be far greater than the differences here stated by Barnes and Gaw. In studying the extremes of these variations, Pütter (1900), for example, found that in a total of 1,100 pulsations observed, the interval between pulsations varied from 6 seconds to 43 seconds; in the same animal, they varied as much as 21 seconds. Frisch (1935) further observed among other facts that swimming, for example, produces a marked decrease in the rate. The decrease begins suddenly when swimming commences and stops suddenly when it stops. Averages for several animals gave the results of 27.5

seconds at rest before swimming, 157.7 seconds swimming, and 28.1 seconds at rest after swimming. Furthermore, the rate varies when animals are at rest, depending upon the time of feeding.

Secondly, it is difficult to apply the Arrhenius equation to the contractile vacuole rate due to this variation. At the present time, we do not know the nature of the reaction or reactions which govern the contractile vacuole discharge. All that we can say is that the filling of the vacuole and its discharge may be the results of numerous types of reactions, of a possible catalytic nature; whether the same causative reactions are affected by deuterium and by temperature cannot be stated.

Finally Barnes and Gaw make no designations as to which of the two vacuoles in *Paramecium* were observed. Fortner (1924) as well as Frisch (1935) have noted that the posterior vacuole contracts more frequently than the anterior one, although in a few animals the reverse was true. Among a group of twenty animals, Frisch shows in his Table I (p. 13) that the variation in the difference of contraction may range from .2 second to 13.3 seconds in cases where the posterior vacuole is contracting the more rapidly.

Since so little is known at present about the function or regulation of the contractile vacuole as a typical but not universal character of the protozoan cell, it does not present itself as a favorable function for the basis of suitable experiments to ascertain the effects of D_2O.

In considering the effects of a high concentration, this protozoan, like other organisms, cannot survive for more than a very brief time in 98 per cent D_2O. In contrast to *Euglena gracilis* (Harvey) an irreversible injury is present.

CONCLUSIONS

1. No indications were found for the stimulation or depression of the fission rate of *Uroleptus mobilis* in 0.44 per cent D_2O during that part of the life cycle which covered about ninety days.

2. On the other hand, with a concentration of 48.1 per cent D_2O both the fission rate and the total longevity were decreased. The degree of the decrease depends to some extent upon the age of the animals since the previous conjugation. Young and old animals survive for only a short time such as thirty days after immersion into 48.1 per cent D_2O; while mature animals live for many days. This shows the necessity of knowing the history and of controlling the experimental material in such organisms as Protozoa. Short exposures to 48.1 per cent D_2O such as for thirty days even with old animals have no harmful effects, as evidenced by the fission rate and the length of life after

return to normal water, whereas long exposures such as eighty days with mature animals result in a decrease in the total longevity.

3. In general, a lowering of the contractile vacuole rate occurs in 48.1 per cent D_2O. The extent of the decrease varies from animal to animal. However, due to the small amount of knowledge upon the exact causative reactions which govern the contraction as well as the normal variation in the rate for different animals, the function is not favorable for study in this respect in this protozoan.

4. Ninety-eight per cent D_2O causes an irreversible injury resulting in immediate death, although there is evidence that some few animals can withstand the effects for a few hours longer than the majority.

BIBLIOGRAPHY

BARNES, T. C., 1934. The effect of heavy water of low concentration on Euglena. *Science,* **79**: 370.

BARNES, T. C., 1935. The biological effects of heavy water. *Am. Jour. Sci.,* **30**: 14.

BARNES, T. C., AND H. Z. GAW, 1935. The chemical basis for some biological effects of heavy water. *Jour. Am. Chem. Soc.,* **57**: 590.

BARNES, T. C., AND T. L. JAHN, 1934. Properties of water of biological interest. *Quart. Rev. Biol.,* **9**: 292.

BARNES, T. C., AND E. J. LARSON, 1934. The influence of heavy water of low concentration on Spirogyra, Planaria and on enzyme action. *Protoplasma,* **22**: 431.

CALKINS, G. N., 1919. Uroleptus mobilis Engelm. II. Renewal of vitality through conjugation. *Jour. Exper. Zoöl.,* **29**: 121.

CALKINS, G. N., 1926. Biology of Protozoa. Lea and Febiger.

CURRY, J., R. PRATT, AND S. F. TRELEASE, 1935. Does dilute heavy water influence biological processes? *Science,* **81**: 275.

FORTNER, H., 1924. Über die physiologisch differente Bedeutung der kontraktilen Vakuolen bei Paramaecium caudatum Ehrenberg. *Zool. Anz.,* **60**: 217.

FOX, D. L., 1934. Heavy water and metabolism. *Quart. Rev. Biol.,* **9**: 342.

FRISCH, J. A., 1935. Contractile vacuole of Paramecium. Regulation and function. Dissertation, The Johns Hopkins University Library. (To be published.)

GREGORY, L. H., 1925. Direct and after effects of changes in medium during different periods in the life history of Uroleptus mobilis. *Biol. Bull.,* **48**: 200.

GREGORY, L. H., 1926. Effects of changes in medium during different periods in the life history of Uroleptus mobilis. *Biol. Bull.,* **51**: 179.

HARVEY, E. N., 1934. Biological effects of heavy water. *Biol. Bull.,* **66**: 91.

PÜTTER, A., 1900. Studien über die Thigmotaxis bei Protisten. *Arch. f. Anat. u. Physiol., Physiol. Abt., Supplbd.,* S. 243. (See Frisch. Original article not read.)

TAYLOR, H. S., W. W. SWINGLE, H. EYRING, AND A. H. FROST, 1933. The effect of water containing the isotope of hydrogen upon fresh water organisms. *Jour. Chem. Phys.,* **1**: 751.

THE STRUCTURE AND FUNCTION OF THE GUT IN SURF PERCHES (EMBIOTOCIDÆ) WITH REFERENCE TO THEIR CAROTENOID METABOLISM[1]

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In examining freshly-caught surf perches (Embiotocidæ) for trematodes, one of us (Young) encountered an interesting yellow or orange pigmentation of the gut, especially in the rectal segment of these fishes. Chemical and spectroscopic studies revealed the carotenoid (specifically xanthophyllic) character of much of this pigment. In freshly-caught specimens of these fish and of the spot-fin croaker (*Roncador stearnsi*) the rectum is frequently but not always deeply pigmented throughout its length to within a few millimeters from the anal opening, ranging in color from a brilliant orange to a deep brown. The upper intestine may also be colored to some extent, but it is the rectum which is most striking.

This observation suggested to us an experimental study of the rôle of the gut in the metabolism of these pigments, and for this purpose we selected *Cymatogaster aggregatus*,[2] a small perch which occurs abundantly in San Diego Bay and elsewhere along the west coast of North America. It has an average weight of 15–20 grams.[3] It is silvery in color with yellow markings on the sides of the body, which alternate with dusky bars.

The description of the gut which follows is based partly on that of *Cymatogaster* and partly on that of *Embiotoca jacksoni* and *Abeona minima.* The structure being very similar in all, the description of one will serve equally well for that of the others.

Several of the standard fixatives were used for the histological work including Bouin, Zenker, Fleming and Carnoy; while paraffin sections were stained mainly in Delafield and Haidenhain's hæmatoxylin, counterstained with eosin, and a few in Giemsa, as recommended by Lee.[4] With the latter stain we obtained some very fine results, especially of the mast cells and of the ganglion cells in the myenteric plexus. Besides studying the vascular supply of the gut in fresh material, we also injected two specimens of *Embiotoca* with India ink, one of which gave a very successful result.

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[1] In this work, Young was responsible for the descriptive and histological portions, while the chemical analyses and spectroscopic observations were made by Fox. The experimental work was carried out jointly.

[2] A number of observations and experiments have also been made on other species, but *Cymatogaster* was the fish mainly used, and the one selected for all the quantitative studies reported here.

[3] Jordan and Evermann give its length as 6 inches but this is much above the average of our specimens, which is about 8–10 cm.

The general anatomy of the digestive tract in surf perches is shown in Fig. 1. The bile duct enters the stomach just behind the pharynx, so that a true stomach is wanting. The rectum forms about one-tenth of the total length of the intestine, and is demarcated from the rest of the latter by a distinct sphincter.

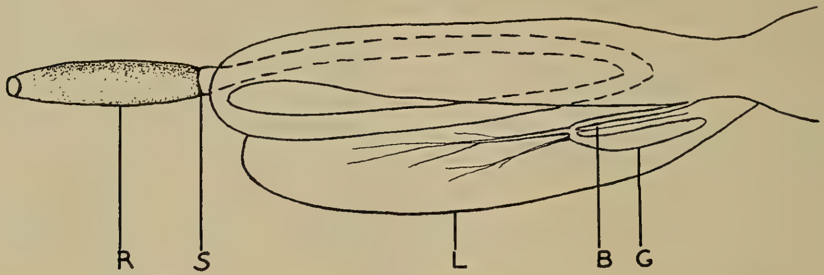

FIG. 1. Digestive tract of *Abeona* × 4. *B,* bile duct; *G,* gall bladder; *L,* liver; *R,* rectum; *S,* sphincter.

Internally the lining of the gut is similar throughout. It is elevated in narrow folds or ridges that follow an irregular, zig-zag course and in general run parallel to the walls of the gut, but whose form and direction is largely determined by the condition of the organ itself. If the latter is filled with food, the folds flatten out and assume a more transverse direction; if the gut is empty the folds heighten and follow more nearly a longitudinal course. At the apices of the angles formed by their bends the folds frequently send out offshoots which may end blindly, or occasionally anastomose with neighboring ridges (Fig. 4).

The blood vessels of the gut have been studied mainly in *Embiotoca jacksoni* instead of *Cymatogaster,* because of the greater ease of injecting them in the larger fish. After penetrating the two muscular layers of the gut wall the vessels branch frequently to form a plexus at the base of the mucosa (Fig. 2) whence smaller twigs extend through the latter to terminate in a very fine plexus at the summit of the folds, just below the epithelium (Fig. 3). In sections prepared from a specimen of *Embiotoca* injected with India ink, these twigs can be followed in

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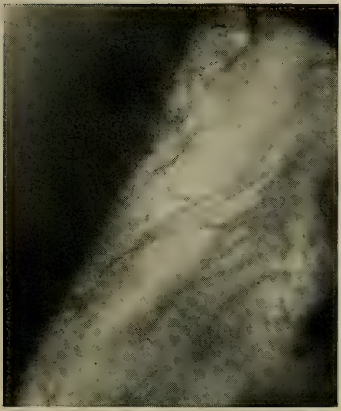
[4] Vade Mecum, Ed. 9, p. 503.



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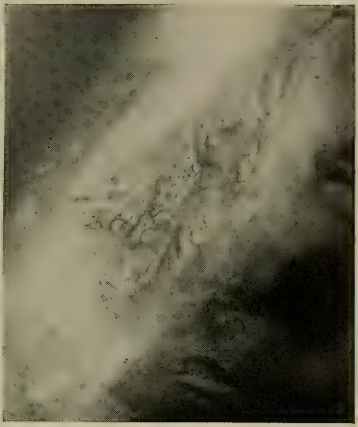

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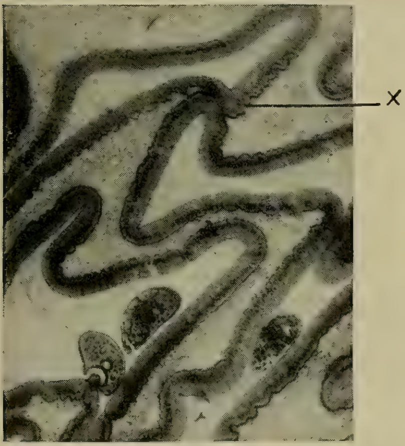

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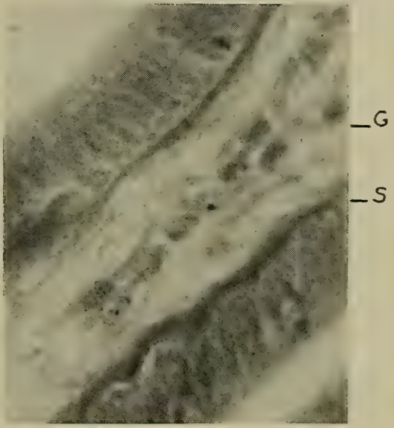

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FIG. 2. External vascular plexus from an injected specimen of *Embiotoca.*

FIG. 3. Internal plexus of same.

FIG. 4. Intestinal rugæ of *Cymatogaster,* from a tangential section. The stratum granulosum is the loose layer between the epithelial folds, while the compactum is the darkly stained line at their base. An anastomosis between two rugæ is shown at *X*. Three trematodes are seen in the lumen between the rugæ. × 130.

FIG. 5. Section of a ruga from the rectum of *Cymatogaster*. *S*, stratum compactum; *G*, stratum granulosum with mast cells. × 560.

sections close to the epithelium but careful search fails to reveal any penetration of the latter by the capillaries. There are several accounts in the literature, however, of the penetration of the epithelium by capillaries in the gut of the Cobitidæ.[5]

The wall of the gut is similarly constituted throughout, so that it is very difficult to tell what region any section represents. The absence of a muscularis mucosæ removes any distinction between mucosa and sub-mucosa, regarding which considerable confusion exists in the literature (see Blake, 1930, and Rogick, 1931).

Greene (1912) divides this layer (mucosa + sub-mucosa) in the intestine of the salmon, where a muscularis mucosæ is absent, in contradistinction to the stomach where it occurs, into three sub-layers: tunica propria, stratum compactum and stratum granulosum. Both of the former authors describe a basement membrane of the epithelium which (see Blake, *loc. cit.,* p. 53) is formed from the tunica propria in the stomach of *Centropristes.*

In our own material basement membrane and stratum compactum are indistinguishable, the latter being well developed and sharply set off from the rest of the connective tissue, or stratum granulosum (Fig. 5). This is so named by Greene because of the presence within it of large numbers of granular cells whose " special and characteristic feature . . . is the presence of the cytoplasmic granules that always crowd them, uniformly filling the cell body " (*loc. cit.*, p. 83). In the salmon these cells are not restricted to the stratum granulosum, but occur also in the stratum compactum, and occasionally in the tunica propria and the sub-mucosa. Greene suggests that these cells form an internal secretion, probably lipase, which is discharged " directly into the surrounding tissue spaces from which distribution takes place " (*loc. cit.,* p. 84).

These cells have been described in detail by Bolton (1933) as mast cells whose " true function seems as yet to be as obscure as that of the mast cell of the higher vertebrates . . . (but which) may play some rôle in connection with the elaboration of zymogen in these organs " (*loc. cit.,* pp. 576–7).

In our material, cells which are undoubtedly the same as those described by these authors occur in considerable numbers scattered through the connective tissue of the mucosa. In some places they occur in groups, in others as isolated cells. They are most abundant in the rectum, but are widely distributed throughout the gut. In places the granules are scattered through the connective tissue, as though set free by the rupture of the cells, but for the most part, the cells are discrete, varying in shape from globular or ovoid to ameboid. Whether they have any relation to metabolism of the carotenoids in the gut, we cannot say.

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[5] See especially in this connection Abolin (1924).

The carotenoids are found in the mucosa of the gut, as is easily demonstrated by washing this tissue with a fine jet of water applied with sufficient force to remove it in patches, leaving it intact elsewhere. Where the latter has been removed the pigment is absent, but is present elsewhere. And when the mucosa begins to break down post mortem the pigment is readily washed out with it. Whether the pigment is restricted to the epithelium, or is present in the connective tissue also we do not know. However, when a brightly pigmented rectum is dissected so as to expose the mucosal folds, the pigment appears as lines or streaks in the latter, and since these are composed mainly of epithelium it is probable that this is the chief, if not the only locus of the pigments.

The source of the pigment is the food. The diet of the surf perches is a varied one, consisting of mussels, clams, barnacles, worms, many kinds of Crustacea and even fish. *Cymatogaster,* on which our experiments were mainly performed, feeds extensively on little green and brown shrimps (*Hippolyte californiensis*) [6] together with a species of *Crangon,* copepods, etc. When brought into the laboratory from the bay the recta are characteristically bright orange in color, while the upper intestine is salmon colored, fading out posteriorly, however, so that the last half centimeter or so is usually colorless, or very pale. The color of the upper intestine is more or less affected by the rich vascular supply of this region, so that in a faintly pigmented gut it is not always easy to determine how much of the color is due to carotenoid and how much to blood pigments. In other surf perches (*Embiotoca,* and *Hyperprosopon*) taken from the surf, and in specimens of *Abeona* which have been kept in aquaria and fed upon various diets, or left unfed, the color of the rectum varies from yellow, through orange to olive or brownish, depending upon the kinds of pigment, derived from food or other sources, or possibly upon chemical changes which the pigments may have undergone.

The rectal pigments which depart in color from pure yellows or orange are not carotenoids, as was demonstrated on several occasions by treating the ground tissue directly with lipoid solvents which failed to extract pigment. Water, or alcohol extracts assumed a pale cloudy yellow tint, but no pigment could be transferred from these solutions to ligroin (Eastman Kodak Co., B.R. 70–90° C.).

By no means all of the available carotenoid pigment in the food is absorbed, for the partly digested food and the feces of fish which have been fed shrimps are always colored, and brightly colored bits of Garibaldi tissue may be found in the feces of fish which have been feeding thereon. Even in a fish whose intestine exhibits no appreciable color the food may retain much of its pigment, as was shown by an *Abeona* whose gut was practically colorless, but which contained the brightly colored remains of a shrimp. Carotenoids may be recovered in considerable quantity from the feces of perches which consume shrimp and other crustacean food. (See below.)

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[6] The authors acknowledge their thanks to Dr. Martin W. Johnson, of the Scripps Institution of Oceanography, for verifying the identity of this shrimp.

QUALITATIVE AND QUANTITATIVE STUDIES OF THE YELLOW PIGMENTS

Several chemical analyses and spectroscopic observations [7] were made of the carotenoid pigment or pigments in each of the following: the entire intestines or the recta alone, the gut contents and feces, the shrimps upon which the perch feed, and the fish bodies minus viscera.

Rectal Segments

A number of these were removed from several specimens (some freshly caught and others having been fed in the aquarium for some days their natural diet of shrimps) and analyzed.[8] Trituration with a little sand and absolute methyl alcohol caused this orange colored tissue to yield its carotenoid material promptly and completely to the solvent, rendering it yellow orange in color. The pigment remained hypophasic, i.e., dissolved entirely in the lower aqueous-alcohol layer when shaken with mixtures of 90 per cent CH_3OH and ligroin, whether the alcoholic solution had or had not first been hydrolyzed for some hours in warm alcoholic KOH. Acidification or alkalization did not alter this characteristic. The pigment was easily transferred from the alcoholic solution (neutralized to preclude the formation of stubborn emulsions if necessary) to ligroin by adding sufficient water and shaking the mixture. It was quantitatively removable from alcohol-free ligroin solutions by adsorption on a column of dry, powdered $CaCO_3$, upon which it exhibited a pale yellow-red band at the top of the column, with a rose-colored band just beneath.

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[7] Spectroscopic observations in this work were made with the large Bausch and Lomb spectrometer No. 2700. Absorption maxima were estimated, and the average of a number of readings taken, in each determination, since the accessory spectrophotometric equipment for securing more precise data was not at hand at the time that this work was done.

[8] In some analyses the whole intestines, from esophagus to anus, were used; in others, the rectal segments only. Often the intestine exclusive of the rectum appeared quite pale and yielded little or no pigment. Even when the absorbent portion of the gut was distinctly pigmented, no differences were detected in the nature of the extracted carotenoid material.

The carbon disulphide solution of the unseparated pigments was deep red orange in color, and exhibited absorption bands whose maxima averaged approximately: I 499.4 mμ, II 471.6 mμ.

Ethereal solutions of the pigments shaken with concentrated HCl remained colorless.

The foregoing properties of the pigment in the intestinal tissue (especially concentrated in the rectum) indicate that it is a carotenoid, or more probably a mixture of two carotenoids judging from the two rings in the $CaCO_3$ column, of a xanthophyllic nature, and unesterified. The absorption spectrum corresponds rather closely to those of taraxanthin and violaxanthin (Zechmeister, 1934; Lederer, 1935) of the $C_{40}H_{56}O_4$ series; the additional fact that concentrated HCl imparted no blue color to ethereal solutions of the pigment indicates that the preponderant carotenoid is not violaxanthin but probably chiefly taraxanthin or a carotenoid indistinguishable from the latter by these various tests.

No attempt was made to separate the adsorbed pigments in the chromatogram, since the total quantity of available pigment was very small, and the paleness of the primary ring indicated a relatively small quantity of this fraction. Furthermore, the spectroscopic picture of the rectal pigment was nearly identical with that of the shrimp xanthophyll, that of the fish skin xanthophyll, and with the body xanthophylls of other species of fish (see Fox, 1936). Why the rectal and shrimp xanthophyll chromatogram showed a red component, while the skin xanthophyll (hydrolyzed) chromatogram consisted in a yellow ring is yet unexplained, but it is possible that the xanthophyll stored in skin is either altered chemically without materially shifting the absorption bands, or rigidly separated from another carotenoid having a very similar spectrum.

Intestinal Contents; Feces

No differences were detected in the nature of the carotenoids of the ingested food removed from the gut and those found in voided feces. Absolute methyl alcohol extracted quantitatively all of the yellow colored fat-soluble compounds. Shaking a 90 per cent CH_3OH solution of the pigments with an equal quantity of ligroin resulted in most of the colored compounds remaining in the lower (alcoholic) layer, with some pigment being transferred to the ligroin phase above. Selective solubility in ligroin is characteristic of carotenes, esterified xanthophylls, free (neutral or acidic) astacene, and certain astacene esters. (See Zechmeister, 1934; Lederer, 1935; Fox, 1936.) That no free astacene was present in the ligroin was demonstrated by adding a drop of strong lye to the aqueous alcohol layer which did not, upon shaking, remove any pigment from the ligroin phase. A portion of the whole extract was hydrolyzed in warm alcoholic KOH, usually for some hours, after which three components were easily separated, whose descriptions follow.

(1) A red substance, insoluble in ligroin or absolute CH_3OH, remained in flocks on the bottom of the flask. When collected on filter paper and washed several times with alcohol and ligroin the material was finely divided and bright red in color, resembling that of ripe tomatoes or red peppers. It remained completely insoluble in neutral ligroin, CH_3OH, or CS_2 but was readily dissolved in any of these solvents if acidified by adding a trace of glacial acetic acid. It exhibited, therefore, the properties of a fat-soluble acidic compound capable of forming a potassium salt which was insoluble in the various organic solvents. The acidified product yielded bright yellow ligroin solutions, salmon-colored CH_3OH solutions, and a beautiful rose pink to magenta color in CS_2. It was hypophasic when shaken with equal quantities of ligroin and 90 per cent CH_3OH. Dilution of the alcohol layer caused the free pigment to be transferred to the ligroin phase. Solutions in ligroin or CS_2 (always freed from the last trace of alcohol and acetic acid) when passed through a $CaCO_3$ column left the pigment quantitatively adsorbed to this salt in a single rose-colored ring at the top of the column. It was readily desorbed, as are the xanthophylls, by ligroin containing a trace of CH_3OH. This pigment was even adsorbed to some extent by pure, dry, crystalline NaCl imparting to this salt a delicate rose tint. It was desorbed by wetting the salt with a little water.

The pigment proved to be rather unstable chemically; when recovered from solvent and stored dry in a partially evacuated chamber, the red color faded completely in a few days. The deep red solution of this rather unusual, acidic carotenoid in CS_2 solution gave a very poorly-defined absorption shadow down in the violet, the average maximum absorption region, taken from numerous readings, lying close to 499.5 $m\mu$, which is somewhat lower than that of astacene (510 $m\mu$ in CS_2 according to Lederer, *op. cit.*). While also acidic, this compound differs markedly from astacene in other ways as well as in its absorption maximum, viz.: its preferential solubility in alcohol when subjected to partition between alcohol-ligroin mixtures; the insolubility of its potassium salt in alcohol (astacene, preferentially soluble in ligroin, can be transferred immediately to the alcohol layer by adding a drop of lye and reshaking (Zechmeister, *op. cit.*)), its instability in air, and its quantitative adsorption from ligroin or CS_2 solutions by $CaCO_3$. Were it not for the acidic character of this red carotenoid, it would correspond rather closely to Lederer's glycymerine, which is neutral, shows a single absorption maximum at 495 mμ in CS_2, preferential solubility in 90 per cent CH_3OH, adsorbability on $CaCO_3$, and other characteristics which liken it to the xanthophyll group of pigments. Until further study may provide more information concerning this interesting pigment, no provisional name will be assigned to it.

(2) When the filtrate from the crop of red flocks was shaken with ligroin, a relatively small quantity of yellow pigment remained in the ligroin layer and was not removable therefrom by subsequent washes with 90 per cent CH_3OH, whether neutral, acidified, or alkalized.

The ligroin solution was freed from alcohol and acetic acid with repeated water washes and then tested chromatographically by passing through a $CaCO_3$ column. No pigment whatever was adsorbed. Transferred to CS_2, after evaporating the ligroin in a current of illuminating gas (to prevent bleaching from oxidation), the solution had an orange color, and exhibited absorption bands whose average maxima lay respectively at: I 511.7 mμ; II 480.9 mμ, which values correspond closely to the first two principal maxima in the visible region for CS_2 solutions of α-carotene (i.e., I 510 mμ; II 478 mμ) and depart somewhat from those of β-carotene (I 521 mμ; II 485 mμ).[9] That it is a carotene is shown by its neutral character, its preferential solubility in ligroin instead of in 90 per cent CH_3OH, and its non-adsorbability on $CaCO_3$. Its absorption spectrum would indicate that the preponderant part of it was closely allied to, if not identical with α-carotene.

(3) The hypophasic (alcohol-soluble) portion of the original filtrate of the hydrolyzed mixture yielded no further pigment to washes of ligroin applied subsequently to the first separation. After transference to ligroin by diluting the alcohol phase, shaking, separating, and finally washing the ligroin solution free from all traces of alcohol, the pigment was quantitatively adsorbed by $CaCO_3$ most frequently as a single yellow band (sometimes as a reddish band) at the top of the column.

The orange colored CS_2 solution of such pigment fractions gave absorption bands with average maxima readings at the following points: I 496.9 mμ; II 467.4 mμ. In its neutral character, hypophasic property, and its quantitative adsorption upon $CaCO_3$, this pigment, like that of the intestine, showed xanthophyllic properties. Also, its absorption spectral bands showed maxima which agreed rather closely with those of the intestinal pigment although the latter's maximal readings were placed slightly more toward the red. While the intestinal pigment always showed two rings, i.e., a yellow, and a red one just below it in the $CaCO_3$ chromatogram, the xanthophyll fraction of the feces and food material in the gut did not always show two such rings, but frequently only the yellow one. The presence of the red ring as well as the yellow, indicating another compound, very possibly depended upon the stage of digestion and relative degree of absorption of the compounds which may have already occurred by the time that the feces or intestinal contents were collected. Since this feature in the chromatogram characterized the xanthophyll of both the intestinal tissue of the fish and the whole shrimps which formed its raw food, and hence the source of the carotenoids, one might have expected to find some such inconsistency in the relative amounts of the xanthophylls in the corresponding fraction of the food boli or feces. The red component in the chromatogram might have been either another xanthophyll or an incompletely hydrolyzed ester, since the esters were shown to be less readily sorbed by $CaCO_3$ and to show various color variations between yellow and red in a chromatogram.

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[9] These figures are for visual spectroscopic readings, but see Smith (1936) regarding photoelectric spectrophotometric methods which give shifts in the positions of absorption bands.

Shrimps

These were gathered from the same locality as that from which the perch were taken, constituted the natural diet of the latter, and were taken from the lot which were fed the experimental animals whose tissues were subsequently analyzed. Two or more species were represented, but the predominant form was *Hippolyte californiensis.* Some two dozen of them were dropped into pure CH_3OH, whereupon they quickly lost their original color, whether brown, green, or dull red, and exhibited bright red patches upon various parts of their bodies. They were ground in a mortar with sand, and yielded all their carotenoid pigments to the alcohol.

All pigment was quantitatively epiphasic, being readily extracted from the alcohol by ligroin; shaking the ligroin solution of carotenoids with additional 90 per cent methanol did not cause any transfer of pigment back to the latter solvent.

After hydrolysis for some hours in warm alcoholic KOH, the shrimp pigments were, as in the extracts of the intestinal contents or feces, resolvable into three components, which were virtually identical in chemical and spectroscopic properties with the respective components of the latter, as follows:

(1) A red, acidic carotenoid, whose potassium salt was insoluble in alcohol, ligroin, or CS_2, but which when free was readily soluble in any of these; hypophasic property in ligroin + 90 per cent methanol systems; quantitative adsorption by $CaCO_3$ as a rose-red ring at top of column; poorly defined absorption band with maximum at approximately 494.5 $m\mu$.

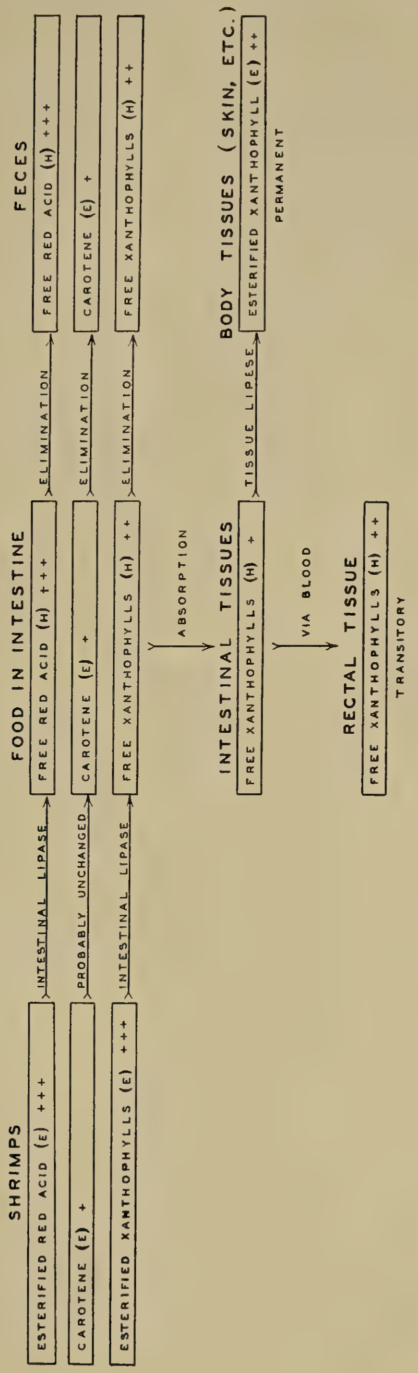
SHRIMPS
ESTERIFIED RED ACID (E) +++
CAROTENE (E) +
ESTERIFIED XANTHOPHYLLS (E) +++
INTESTINAL LIPASE
PROBABLY UNCHANGED
INTESTINAL LIPASE
FOOD IN INTESTINE
FREE RED ACID (H) ++
CAROTENE (E) +
FREE XANTHOPHYLLS (H) ++
ELIMINATION
ELIMINATION
ELIMINATION
FECES
FREE RED ACID (H) +++
CAROTENE (E) +
FREE XANTHOPHYLLS (H) ++
ABSORPTION
INTESTINAL TISSUES
FREE XANTHOPHYLLS (H) +
TISSUE LIPASE
BODY TISSUES (SKIN, ETC.)
ESTERIFIED XANTHOPHYLL (E) ++
PERMANENT
VIA BLOOD
RECTAL TISSUE
FREE XANTHOPHYLLS (H) ++
TRANSITORY

FIG. 6. The fate of shrimp carotenoids ingested by the surf perch *Cymatogaster aggregatus*. (*E*) denotes that the pigment is *epiphasic* and, excepting in the case of carotene, esterified; (*H*) denotes that the pigment, having been hydrolyzed, is *hypophasic*. The crosses represent, in the conventional manner, not absolute quantities but merely approximate relative concentrations of pigments (D. L. Fox and R. T. Young (Drawn by C. W. Watson)).

(2) A small quantity of an epiphasic carotenoid, unadsorbed by $CaCO_3$, and with absorption bands at approximately, I 513.1 mμ; II 477.7 mμ.

(3) A hypophasic xanthophyll component quantitatively adsorbed by $CaCO_3$ in a yellow ring nearly at the top, and a faint rose ring just beneath it. The reddish orange CS_2 solution of this pigment gave absorption bands with maxima at the approximate average positions: I 500 mμ; II 469.1 mμ; which agree rather closely with the respective absorption bands of the xanthophyllic pigment extracted from the gut tissue and with the corresponding component of the shrimp tissue in gut or feces. Like these pigments, also, this one from the shrimps gave no color reaction with concentrated HCl.

It is important to note that while the xanthophyllic pigments of the gut contents or feces of the fish, as well as those of the intestinal and rectal mucosa were free (i.e., unesterified, hence preferentially soluble in 90 per cent CH_3OH), the corresponding components in the shrimps which formed the original food and from which the fish pigment was derived were completely esterified, manifesting this property by complete preferential solubility in ligroin. Similarly, the red acidic carotenoid must have undergone hydrolysis in the gut, through the agency of lipase, for, whereas in food boli from the gut or in fecal material this pigmentary constituent was completely hypophasic as were the xanthophylls, the same compound was completely epiphasic in the original living shrimp tissue. It is possible that in nature the red acidic carotenoid may be conjugated with the alcoholic xanthophylls.

The behavior of this red acidic carotenoid recalls that of astacene, which may exist, according to Kuhn and Lederer (see Lederer, 1935; Zechmeister, 1934) as epiphasic esters, preferentially soluble in petroleum ether, or as hypophasic esters, more soluble in 90 per cent CH_3OH, in different tissues of the Norwegian lobster.

Fish Bodies, Exclusive of Viscera

Bodies of whole eviscerated perch which had been fed shrimps for some days in the aquarium were chopped up with scissors and then ground with alcohol in a bronze ball mill (Sumner and Fox, 1935*a*) until the material was reduced to a fine pasty consistency, the proteins being denatured and relatively dehydrated by the alcohol, and the carotenoid rendered subsequently easily extractable. The most striking observation was that the pigment was hardly extracted at all by methyl alcohol at this stage, but was quantitatively removed by ligroin. Needless to add, it was completely epiphasic. Also interesting was the observation that the xanthophyll ester (see below) seemed to be more resistant to hydrolysis than were those which occurred in the original shrimps consumed by the fish. This may have been due either to different fatty acids having been involved in the esterification, or to more hydroxyl groups having been esterified in the fish body pigment, for the xanthophyll itself was by all tests identical with the principal one found in the food and rectum.

The spectrum of the epiphasic, unhydrolyzed compound in CS_2 showed two strong absorption bands with average maxima at I 497 mμ; II 468.6 mμ.

After hydrolysis in alcoholic KOH, the pigment was quantitatively hypophasic, and was completely adsorbed from ligroin solutions by $CaCO_3$ as a yellow layer at the top of the column. The orange-colored CS_2 solution showed absorption bands with average maxima at I 497.5 mμ; II 466.8 mμ. It will be noted that the xanthophyll, apparently a single compound, in the tissues (especially skin and fins) of the perch showed, after hydrolysis, the same chemical properties as did the xanthophylls from the gut and rectum and the xanthophyll fraction from the shrimps, except that the latter two pigments showed a secondary reddish adsorption ring in the $CaCO_3$ chromatogram. The two respective spectral absorption maxima of the xanthophyll pigment from all three sources are in close agreement; confirming the conclusion that we are dealing with a single carotenoid as the sole pigment in the skin and other non-visceral tissues of the fish, and as the certainly preponderant xanthophyll in the rectum and in the original shrimp tissue.

Having determined the nature of the xanthophyllic pigments in the shrimps taken from natural surroundings, the same while undergoing digestion in the intestine of the perch, those in the rectal segment, and the eviscerated whole body of the perch, the same question as initiated the investigation still confronted us: what if any is the function of the rectal segment with regard to the metabolism of the carotenoid, and what is the probable fate of the carotenoid which is found temporarily stored in this structure?

Several feeding experiments were performed in the laboratory with *Embiotoca, Cymatogaster* and *Abeona,* using shrimps (mainly *Hippolyte*) sand crabs (*Emerita analoga*) and minced flesh of the Garibaldi (*Hypsypops rubicunda*) as food. The latter, as has recently been shown (Sumner and Fox, 1935*a, b;* Fox, 1936), abounds in xanthophyll. (Chemical and spectroscopic data indicate, furthermore, that the xanthophyll of *Cymatogaster* skin is identical with that of the brilliant Garibaldi, although far less copious).

These experiments were undertaken in order to trace the pigment in the gut of the fish. A detailed account of all of them would be not only wearisome, but somewhat confusing, since individual fish differed rather widely in their response to this feeding. Suffice it to say that in general the pigment appeared in the intestine, with exception of the last half centimeter or so, shortly after feeding, to disappear within a day or two after feeding was ended. The recta did not take up the carotenoid pigment extensively in many of these experiments, and it is difficult to decide whether the color which was observed in them was due to the experimental feeding or to residual pigments of catabolic origin, or from the food consumed in nature. In one experiment an *Embiotoca* which had been fed *Emerita* on two occasions and was killed five days after the first feeding, and one day after the second, contained almost no color in the rectum, while in another experiment a *Cymatogaster,* which had been fed shrimps for several days previously had no apparent color in the rectum. Occasionally, however, the recta were brightly colored in fish which had been previously fed on carotenoid-rich food.

While the intestine proper is undoubtedly a digestive and absorptive organ, secreting enzymes which hydrolyze both the esterified xanthophyll and the combined red, acidic carotenoid, subsequently selectively absorbing the free xanthophyll, the possible function of the rectum with regard to the carotenoid is still uncertain. It may be an organ for (1) absorption, although this possibility seems very doubtful, (2) storage, (3) elimination, (4) destruction (oxidation?) of the pigment, or some combination of these.

It was of interest to determine whether or not the rectum might be a kind of storage organ for xanthophyll for the re-supply of other body tissues which might utilize it. Accordingly, a group of *Cymatogaster* from San Diego Bay were held in a laboratory aquarium for some weeks, several of their number being sacrificed at stated intervals of days, eviscerated, weighed, and the body carotenoid quantitatively extracted and determined in the regular way. At the same time the whole gut was carefully examined and its appearance with regard to pigmentation recorded. The quantities of rectal xanthophyll were analyzed for the first two lots of fish killed, but in later examinations the amounts of carotenoid in rectal tissue were so small that quantitative determinations were impossible.

The perch refused proffered food, and therefore consumed nothing except a few minute copepods which they obtained from the sea water, from the beginning (January 14) to the end (February 18) of the experiment.

The ligroin solution of extracted pigment was, as in previous work (Sumner and Fox, 1933) brought in each case to a volume equal (in

TABLE I

Date killed	Serial Number	Days kept	Number and sex of fish	Recta	Weight in grams	Photometer reading	Xanthophyll (mg. per 100 gr. tissue*)
Jan. 15.......	C_1	1	4 ♀	Bright yellow orange	58.3	60.1	0.27
Jan. 15.......	C_2	1	2 ♀	" " "	29.6	60.2	0.27
Jan. 15.......	C_3	1	2 ♂	" " "	27.5	59.8	0.27
Jan. 14.......	C_4 (stored in refrig. until Jan. 17)	0	3 ♀	" " "	54.4	61.0	0.26
Jan. 18.......	C_5	4	4 } 5 ♀, 3 ♂	Paler and variable	59.8	61.5	0.26
Jan. 18.......	C_6	4	4	" " "	60.2	58.6	0.29
Jan. 24.......	C_7	10	3 (2 ♀, 1 ♂)	Pale, one brown	50.4	56.2	0.30
Jan. 31.......	C_8	17	4 (3 ♀, 1 ♂)	Pale	68.7	53.0	0.34
Feb. 8.......	C_9	25	1 ♀ (large, gravid)	Pale	45.1	64.7	0.22†
Feb. 18.......	C_{10}	35	2 (very small)	Pale	7.2	53.8	0.33
Jan. 15.......	C_1, C_2, C_3		8 recta		(0.4) ‡	62.4	2.5
Jan. 18.......	C_5, C_6		8 recta		—— §	81.4 (far paler)	1.0

* Calculated on a basis of the standardization of the Ives photometer with sunflower xanthophyll (largely lutein, provided by Dr. J. H. C. Smith and Dr. H. H. Strain of the Carnegie Institution of Washington, Stanford University). Xanthophylls from this source have absorption bands placed farther toward the red, having therefore a higher tinctorial power than the fish xanthophylls described by the present authors (also by Sumner and Fox, 1933, 1935*a*, 1935*b*, and Fox, 1936). Hence the determined values for concentrations of the latter xanthophyllic pigments are consistently somewhat lower than the true values, but these departures must be virtually uniform, since we were dealing with the same xanthophyll in all the fishes investigated to date.

† The rather well-developed young in this large, gravid female were discarded along with the viscera before analyses. It is very probable that this species, in common with most carotenoid-containing animals, transfers considerable quantities of the pigment from the body to eggs and young. This would account for the low figures in the particular specimen.

‡ I. e., 8 recta weighing 0.4 gram in the aggregate, excised from 8 fish, weighing 115.4 grams in the aggregate.

§ These recta were not weighed, but came from 8 fish weighing 120.0 grams.

ml.) to twice the weight (in grams) of the fish tissue extracted, giving uniform ratios of 2 ml. per gram throughout the series. In analyzing the recta, the pigment from the total (approximately 0.4 gram) of each lot was dissolved in 8 ml. ligroin, giving a ratio of 20 ml. per gram.

Table I presents a resumé of the experimental findings.

Observations made at the time that each lot of fish was sacrificed, revealed a progressive decrease in the pigmentation of rectal tissue, as shown by the difference between the xanthophyll content of the eight fishes' recta on the first day, and that of the eight sacrificed three days later. The carotenoid pigment found in the intestine proper was manifestly being constantly carried away in the blood, while that encountered in rectal tissue remained for longer intervals.

The apparent increase in body xanthophyll, noticed at about the ninth day, was without doubt due to a mere increase in relative quantity through loss of body weight following the refusal of food by the animals; the esterified xanthophyll of the skin tissues was evidently less readily catabolized than other body substances. Previous quantitative work by Sumner and Fox (1935*a*) showed that a different species of fish, *Fundulus parvipinnis,* which stores the same xanthophyll in esterified form in the skin and other body tissues, underwent no absolute loss of pigment when kept in the laboratory upon a carotenoid-free diet for three months.

Cymatogaster, when taken from their natural surroundings, gorged with food rich in carotenoids (including the xanthophyll which they themselves store), showed values of only 0.26–0.27 mg. xanthophyll per 100 grams of non-visceral body tissue, while the rectal tissues were usually brilliantly pigmented and the " stomach " colored to a less degree with carotenoids. Such animals kept for some days without food in laboratory aquaria maintained their relative quantity of body xanthophyll, while their rectal and intestinal carotenoid fell to nil. Should the rectal segment play the rôle of a storage organ for maintaining the supply of body xanthophyll, we might expect a prompt drop in the latter after the disappearance of the pigment from the former. Since the quantity of body xanthophyll neither builds up, beyond a rather low maximum, in nature when the supply in the food is plentiful, nor drops from this value in captivity when food is not consumed, it would appear certain that the uncombined xanthophyll temporarily stored in the rectal mucosa is not serving the purpose of resupplying the other tissues with pigment.

Since the rectal xanthophyll did, nevertheless, disappear rather soon, it seemed possible that it was being either (1) destroyed in situ, perhaps through the agency of an oxidase, (2) carried elsewhere by the blood, and broken down into colorless compounds, or (3) secreted into the lumen of the rectum and subsequently eliminated.

To test the first possibility, several experiments were performed, using mixtures of aqueous extracts of isolated recta which had been preserved at freezing temperatures (although not sufficiently low to freeze the tissues), and both free and esterified xanthophyll in colloidal dispersion. A brief account of the experiments follows: 15 recta (nearly all of them distinctly red in color), stored for a few days at about —4° C. (not frozen), weighing about 0.2 gram, were triturated in a mortar with sand and phosphate buffer at pH 7.0. This pH was adopted because water extracts of *Cymatogaster* recta had shown pH values close to this value. Buffer solution was added to the extract to bring the volume to 10 ml. The solution was passed through a sintered-glass filter-funnel.

Free xanthophyll from *Cymatogaster* tissues was prepared by hydrolyzing the extracted esters. Whether the free or the esterified xanthophylls were used, the ligroin solvent was driven off in a current of gas, and the pigment residue was shaken vigorously with 10-ml. buffer (pH 7.0) solution to give a kind of emulsion. Equal volumes (1.5 ml.) of recta extract (previously boiled in the controls) were added to each 10-ml. system; 3 drops of toluene were introduced as bacteria-preservative, and the systems were allowed to stand at room temperatures in the dark.

At the end of 72 hours, the free xanthophyll was quantitatively recovered from both systems, ligroin solutions of each reduced to the same volume, and photometric measurements were taken in the usual way. The amounts of xanthophyll in both the experimental and the control were identical.

The corresponding xanthophyll ester systems were left for 117 hours, at the end of which time no diminution of pigment in the experimental as compared with the control had occurred; nor had the ester been hydrolyzed by the recta extract.

A similar experiment, using pH 7.0 buffer, and adding a few drops of olive oil to each vessel as an emulsifacient, keeping the systems at 37–38° C. for 21 hours, revealed no difference in quantity of pigment remaining in experiment and control.

Thus no oxidase for the destruction of xanthophyll was demonstrated in extracts of rectal tissue.

Other tissues of the body were not tested for xanthophyll oxidases in this way.

It should be said that red or orange-colored recta, stored at room temperature either (1) in diluted, toluene-preserved sea water, (2) under ligroin or (3) directly under inert, colorless, heavy oil containing toluene as a preservative, were observed to lose their bright pigmentation in two or three days, and not by giving it up to the supernatant liquid. We are hardly prepared to state, however, that this was an oxidation hastened by an *enzyme,* since unesterified xanthophyll had been observed to fade about as readily *in vivo* (as demonstrated) or when extracted and stored in ligroin solutions.

The question next investigated was whether or not the pigment might be secreted by the rectal mucosa into the lumen of the organ, to be subsequently eliminated with the feces. A catch of *Cymatogaster* taken March 20 were brought to the laboratory and kept for about 24 hours, to permit them to expel all their feces. Animals sacrificed for examination at the end of this interval had red to orange-colored recta, but had voided all fecal masses. Thin pieces of wire whose ends were wound with cotton were carefully inserted into the rectum, gently revolved once or twice, and withdrawn. The cotton ends of these probes were still white save in one or two instances in which the tips were tinged with bits of yellowish mucus from the gut.

To apply a more rigid test, cotton plugs were inserted with forceps, one into the rectum of each of a dozen *Cymatogaster,* and the fish were kept in the laboratory aquaria overnight. The seven fish which were still living when examined in the morning had, with the exception of two which had expelled their plugs, retained the cotton in their recta for intervals ranging from 16 to 21 hours.

All five fatalities were females, gravid with young. The significant findings of the experiments are as follows: Notwithstanding the fact that all intestines were without food or feces, and that all save two rectal segments had faded from red or orange to pale yellow or white, all cotton plugs (including the two which had been expelled) remained colorless with the exception of two which were taken from dead animals, and showed yellow tinges. The dead group, unlike the living, all showed yellow mucus in the intestinal tract due to incipient sloughing following death. If in nature the rectal mucosa secreted its xanthophyll into the lumen, it would seem certain that the cotton plugs must have shown at least traces of yellow color. Since the plugs taken from the living fish (and from all save two of the dead) were entirely devoid of color, we conclude that the rectum does not eliminate xanthophyll by secreting it into the lumen.

Whatever may be the reason for the accumulation of free xanthophyll in the rectal mucosæ of perches which consume great quantities of shrimps, we are inclined to the belief that the tissue obtains its xanthophyll from the blood, and not by absorbing it directly from fecal matter in the lumen, for the following reasons:

(1) While the structure of the mucosa, both macroscopically and microscopically, is essentially the same throughout, the 5 mm. or so of the intestine just above the rectum is usually, though not always, colorless or nearly so. Were absorption continuous throughout the tract, there should not be, as there is, a distinct segment above the rectal sphincter usually devoid of color.

(2) The rectum may retain its bright orange color for several days after the fish have been brought into the laboratory and starved, while the rest of the intestine quickly loses it.

(3) In laboratory specimens of *Cymatogaster* or *Abeona* which have been recently fed carotenoid pigment in Garibaldi skin, shrimps, and *Emerita,* the upper intestine nearly as far back as the anal sphincter, characteristically assumes a more or less distinct (in some cases very pronounced) yellow or salmon color, while the rectum seldom does so, even in specimens in which the intestine, including the rectum, is full of food.

The frequent pigmentation of the rectum in nature, and its comparatively rare coloration in specimens fed in the aquaria are probably explicable as due to what we may call a " super-saturation " of the body of the fish in the former, as compared to its " under-saturation " in the latter situation, i.e., xanthophyll is, in the former case, supplied in such quantities and absorbed at such a rate as to exceed the rate of its destruction or elimination, hence it accumulates in blood probably up to a certain level of concentration and any surplus is then deposited in the rectal mucosa. In the laboratory, fish apparently did not usually consume sufficient xanthophyll-rich food, or at any rate did not absorb sufficient quantities of xanthophyll from it to build the concentration of the pigment in blood up to the point where it was deposited in the rectal mucosa pending destruction. In this connection, we are reminded of the yellowing of the shanks, ears, egg-yolks, and body fat of poultry fed upon a diet rich in xanthophyll (Palmer, 1922), the deposition of certain xanthophyllic carotenoids in the feathers of canary birds (Brockmann and Völker, 1934), the increase in the yellow color of cream and butter from cattle fed upon carotene-rich food, and finally the infrequently occurring condition in humans known clinically as xanthosis or carotenemia, in which carotenoids ingested in great quantities in the food are not disposed of rapidly enough by the body to preclude their deposition in the skin, which assumes a distinctly yellow color. The color disappears rather shortly after carotenoids are withdrawn or included more sparingly in the diet (Palmer).

The provisionally proposed paths of various carotenoids ingested in shrimps by *Cymatogaster* are given in summary form in Fig. 6.

SUMMARY

1. A yellow, orange, or red pigmentation of the rectal segment of the intestine in certain surf perches is described and illustrated.

2. The carotenoid pigments of the shrimps consumed by the perch *Cymatogaster aggregatus,* the xanthophyll of the fishes' intestines and recta, and the xanthophyll ester, mainly of the skin, are described with respect to chemical and spectroscopic properties.

3. Carotene (apparently mainly the α-isomer), a red acidic carotenoid (not astacene), and a xanthophyll (or a mixture of two xanthophylls which were not separated) similar in chemical and spectral absorption properties to taraxanthin, are found in the shrimps (mainly *Hippolyte californiensis*). The acidic carotenoid and the xanthophyll(s) are present as epiphasic esters.

4. Both the red acid and xanthophyll are hydrolyzed and rendered hypophasic in the alimentary tract of *Cymatogaster,* but the xanthophyll(s) are apparently the only carotenoids absorbed by the intestine.

5. The carotenoid pigments of the rectal mucosa consist of the same xanthophylls as those in the shrimps, but unlike the latter, are stored in this tissue in unesterified condition. When the fish are not given food containing a supply of the carotenoid, the pigmentation of the rectal segment fades rather soon and may disappear completely in a few days.

6. The xanthophyll stored in the skin and perhaps in certain other tissues of the perch is re-esterified, and appears to be a single xanthophyll. It is small in quantity (0.26 to 0.27 mg. per 100 grams of wet, eviscerated fish tissue) and constant, whether the fish are gorged with shrimps or starved for some days or weeks in the laboratory.

7. Observations and experiments lead the authors to offer the following provisional conclusions regarding the metabolism of the xanthophylls of rectal tissue:

a. The carotenoid material is not absorbed directly from the lumen of the rectum by the rectal mucosa, but is taken up by the mucosa of the intestine proper, and carried by the blood to the rectal tissue; when present in the blood in concentrations which exceed some threshold value, the xanthophyll pigment accumulates in the rectal mucosa;

b. The rectum does not serve as a temporary storehouse to replenish the supply of skin xanthophyll, which is esterified and seems to be relatively stable and permanent;

c. It does not secrete the pigment into the rectal lumen to be eliminated with the feces;

d. It is apparently not an organ specially equipped with an oxidase enzyme for destroying the xanthophyll;

e. Its function may be that of a temporary repository of unesterified xanthophyll, which readily loses its pigment when a xanthophyll-rich diet is withheld.

8. Whether this excess xanthophyll is gradually excreted through channels other than the anus, or whether it is gradually destroyed (oxidized?) in the blood, the authors are unprepared to decide at the present time.

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LITERATURE

ABOLIN, L., 1924. Über den Einfluss der maximalen Darmatmung auf den histologischen Bau des Enddarms des Schlammpeizgers (Misgurnus fossilis). *Biol. Zentralbl.*, **44**: 433.

BLAKE, I. H., 1930. Studies on the comparative histology of the digestive tube of certain teleost fishes. I. A predaceous fish, the sea bass (Centropristes striatus). *Jour. Morph.*, **50**: 39.

BOLTON, L. L., 1933. Basophile (Mast) cells in the alimentary canal of salmonoid fishes. *Jour. Morph.*, **54**: 549.

BROCKMANN, H., AND O. VÖLKER, 1934. Des gelbe Federfarbstoff des Kanarienvogels und das Vorkommen von Carotinoiden bei Vogeln. *Hoppe-Seyler's Zeitschr.*, **224**: 193. *Physiol. Abstr.*, **20**: 81 (1935).

FOX, D. L., 1936. Further studies of the carotenoids of two Pacific marine fishes, Fundulus parvipinnis and Hypsypops rubicunda, and of a marine annelid, Thoracophelia sp. *Proc. Nat. Acad. Sci.*, **22**: 50.

GREENE, C. W., 1912. Anatomy and histology of the alimentary tract of the King Salmon. *Bull. U. S. Bur. Fish.*, **32**: 73.

JORDAN, D. S., AND W. B. EVERMANN, 1898. The fishes of North and Middle America. *Bull. U. S. Nat. Mus.*, No. 47.

LEDERER, E., 1935. Les Carotenoides des Animaux. Hermann et Cie., Paris.

LEE, A. B., 1928. The Microtomist's Vade Mecum. Blakiston, Philadelphia.

PALMER, L. S., 1922. Carotinoids and Related Pigments, A. C. S. Monograph Series. Chemical Catalog Co., Inc., New York.

ROGICK, M. D., 1931. Studies on the comparative histology of the digestive tube of certain teleost fishes. II. A minnow (Campostoma anomalum). *Jour. Morph.*, **52**: 1.

SUMNER, F. B., AND D. L. FOX, 1933. A study of variations in the amount of yellow pigment (xanthophyll) in certain fishes, and of the possible effects upon this of colored backgrounds. *Jour. Exper. Zoöl.*, **66**: 263.

SUMNER, F. B., AND D. L. FOX, 1935*a*. Studies of carotenoid pigments in fishes. III. The effects of ingested carotenoids upon the xanthophyll content of Fundulus parvipinnis. *Proc. Nat. Acad. Sci.*, **21**: 330.

SUMNER, F. B., AND D. L. FOX, 1935*b*. Studies of carotenoid pigments in fishes. II. Investigations of the effects of colored backgrounds and of ingested carotenoids on the xanthophyll content of Girella nigricans. *Jour. Exper. Zoöl.*, **71**: 101.

ZECHMEISTER, L., 1934. Carotinoide. Julius Springer, Berlin.

EFFECTS OF MECHANICAL DISTORTION ON THE STRUCTURE OF SALIVARY GLAND CHROMOSOMES

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It has been maintained by several authors[1] that the giant salivary gland chromosomes of Diptera are compound and that the striations or "threads" to be seen in the fixed chromosomes provide visible evidence of this compound structure. On such a view the "threads" are considered to represent chromonemata, and it is implied that these threads or chromonemata have a fixed size limit and that chromosome enlargement beyond this limit is regularly accompanied by division of the original threads. Without attempting to oppose this view as a theoretical conception, we have in earlier papers[2] presented evidence indicating, (1) that the visible striations or strands under consideration represent distortion effects rather than real threads or chromonemata, and (2) that if multiple chromonemata are present they are probably invisible and more numerous than the striations.

In those papers both chemical and mechanical distortion of the living materials have been considered briefly, and emphasis has been laid on the fact that whereas the striations or "threads" should be most conspicuous in chromosomes which have not been mechanically distorted, their prominence is actually proportional to the amount of twisting or stretching which the chromosomes have undergone. The present account is devoted to further consideration of the effects of mechanical distortion, especially those resulting from adhesions between different chromosome regions.

It is desired to emphasize the following five features here. (1) The protoplasm of these chromosomes is highly viscous and may be stretched into long, delicate strands or "threads." (2) These strands or "threads" of chromosome material may extend laterally or in other directions from a chromosome under suitable conditions of distortion, and may connect with and be indistinguishable from the longitudinal striations referred to above. (3) The chromatic materials appear to be more viscous and more tenacious than the achromatic. Apparently they are more resistant to distortion than the achromatic materials, but may be greatly attenuated if subjected to sufficient tension. (4) By means of mechanical distortion local rearrangements of materials within a chromosome may be produced which result in striations having the same characteristics as the longitudinal striations, but which are evidently artifacts. (5) The number of strands or striations may vary widely in adjacent regions of a chromosome, and vary in such a way as to indicate that they are artifacts rather than a reflection of preexistent structures in the living chromosome.

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[1] See Koltzoff, 1934; Bridges, 1934; Koller, 1935; Muller and Prokofieva, 1935; Bauer, 1935.

[2] Metz and Gay, 1934, 1935; Metz, 1935*a, b;* Doyle and Metz, 1935.

These features will be illustrated by a few examples from a large number available. All are from fully developed glands of *Sciara ocellaris* Comst. The accompanying drawings were all made from aceto-carmine preparations, with the exception of Fig. *2a,* which is from an osmic-aceto-carmine slide. All the figures except the sketch shown in Fig. 1*b* were made by drawing with India ink directly on photographs, and then bleaching the prints. They are necessarily schematized somewhat, since the photographic details cannot be reproduced in plain black and white; but the features under consideration show even more clearly in the photographs than in the drawings. Only features of present interest have been included. It is planned to publish the photographs in a later paper. The drawings were made at a magnification of 3,000 ×, and are reduced to approximately 2,000 × in publication. Acknowledgment is made to Mrs. Elizabeth Gay Lawrence and Mrs. Arlene DeLamater, respectively, for assistance in making the slides and the drawings.

The first-mentioned feature is illustrated in Fig. 1. One localized region of the chromosome shown in this figure was so greatly stretched, in making the smear, that the material became attenuated into a single delicate strand. The delicacy and continuity of this strand show how cohesive the chromosomal constituents are and how greatly the material may be stretched. This single strand exhibits thickenings or " granules " which represent the remains of thickenings or " granules " in the " discs " before the stretching occurred. (It is to be remembered that the material was coagulated by fixation before being subjected to mechanical distortion.)

The upper part of this figure also illustrates the fourth feature mentioned—a rearrangement of materials within the chromosome, and the production of striations by distortion. This aspect will be made more evident by examination of the outline sketch (Fig. 1*b*) showing how the chromosome has been distorted. An adhesion has been formed between one end of the chromosome, at *a,* and the side of the chromosome, at *b*. The distorted region (*c*), therefore, is being held in position to some extent by this adhesion. The transverse bands or discs are in normal position a short distance from this distorted region, but show some distortion in the region marked *d*, and progressively greater distortion in regions *e* and *f*, the latter being the region shown in the larger drawing. In this tapering region, from which the single strand extends, the materials of the chromatic discs, together with the intervening achromatic materials, have been rearranged by the tension so as to produce granular striations (separated by rows of alveoli) focusing on the point of tension. It seems clear that these striations do not represent preëxistent structures, either longitudinal or transverse. The evidence also supports other lines of evidence in indicating that the inter-alveolar, chromatic material is more viscous than the alveolar, achromatic material.

An illustration of the effects of moderate stretching is shown in Fig. 2 and an intermediate condition between this and the one just described is shown in Fig. 3. The region shown in Fig. 2*b* has been stretched only slightly—just enough to elongate the droplets or vacuoles so that they appear hexagonal in optical section. The " honeycomb " structure is conspicuous in cases like this when enough chromatic material is distributed around the vacuoles to reveal them distinctly. With other types of treatment (see Metz, 1935*b*) the chromatic material may be more definitely restricted to the transverse discs, and the outlines of the vacuoles correspondingly less distinct. The region shown in Fig. 2*a* has been stretched a little more. Distortion here has been sufficient to obliterate the more delicate transverse bands, but not enough to obscure the heavier ones or to destroy the internal organization. It seems clear from our observations that the " longitudinal striations " observed in more severely stretched chromosomes are derived from the

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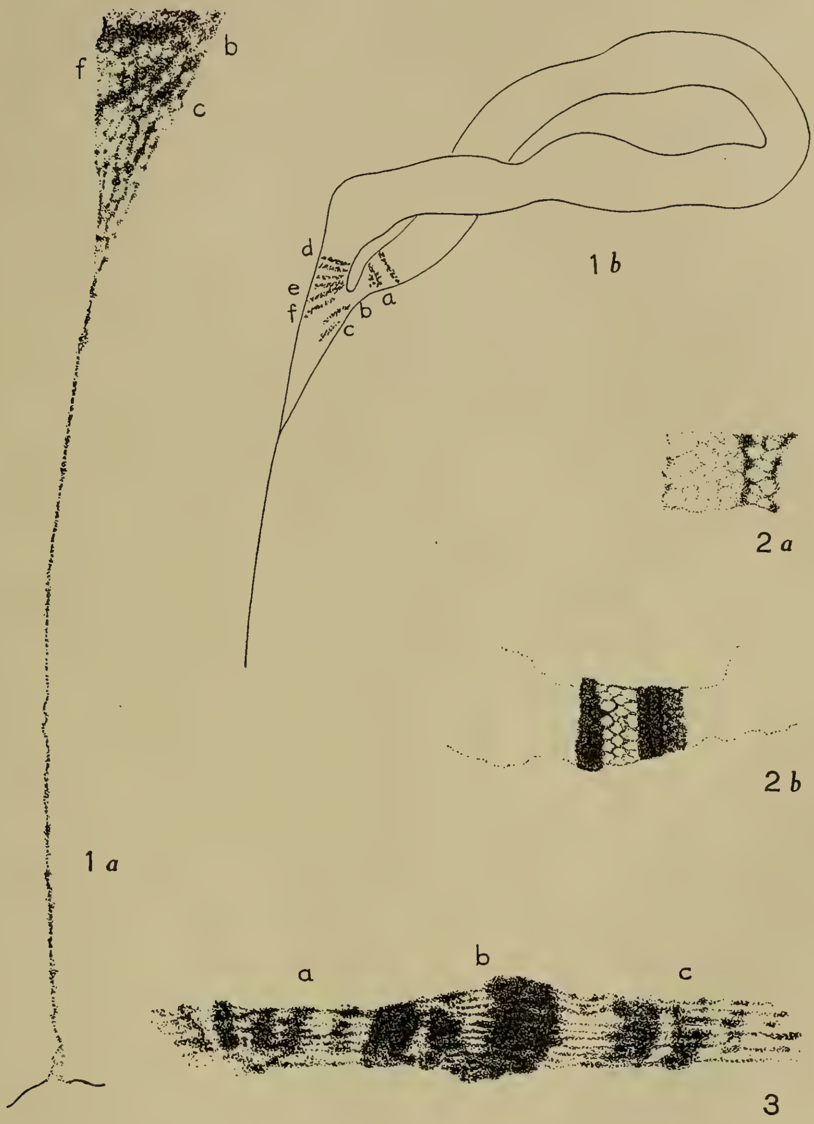
EXPLANATION OF FIGURES

FIGS. 1–3. Illustrating the effects of different amounts of distortion on salivary gland chromosomes.

FIG. 1. The chromosome shown here was pulled almost in two, the two parts remaining connected by the delicate strand shown in the figure. The lower end of the strand is apparently connected with the rest of the chromosome, but the latter is entangled with several others and is not represented in the figure. The wavy outline of the strand in the lower part is not due to lack of tension, but to achromatic droplets or vacuoles, around which it extends. See text for further details. 1*a*, detailed drawing. 1*b*, outline sketch.

FIG. 2. Two regions, slightly stretched, showing the distorted alveolar structure which appears in the form of a network at any one optical level, or a honeycomb when considered in three dimensions. *a* from an osmic-aceto-carmine preparation; *b* from an aceto-carmine preparation.

FIG. 3. Severely stretched portion of a chromosome in which the more delicate structures have been entirely disrupted and the coarser ones disarranged. The finer bands have disappeared and the chromatic material has been pulled out into striations or strands, as described in the text.


b
f
c
1 *a*
d
e
f
a
b
c
1 *b*
2 *a*
2 *b*
a
b
c
3

FIGS. 1–3

lines observed in moderately distorted cases like those shown in this figure. These lines, however, form a network at any one focal level. No discrete, continuous lines of thread-like nature are visible. The structure appears definitely to be honeycomb-like when considered in three dimensions. Comparative study of material fixed in different ways indicates that the compartments of the honeycomb represent the achromatic droplets or vacuoles seen in less distorted material between the cross bands or extending through the cross bands if the latter are represented by granules.

The short lines extending longitudinally between two successive cross bands (discs or rows of granules) apparently represent chromatic material lying between the droplets or vacuoles which occupy this space. Such chromatic material presumably flowed out from the chromatic discs or else became separated from the achromatic material in the process of coagulation. In any event it does not seem to represent threads, but rather to represent the walls of the droplets or vacuoles. These features will be considered more in detail in another paper.

With further stretching the structure just considered becomes more and more distorted, as indicated in Fig. 3. The achromatic material appears to be the first to become disrupted, perhaps because it is in the form of separate droplets. The more delicate parts of the network or honeycomb become broken up, and most of the chromatic material at least becomes drawn into longitudinal striations, with traces of the original type of structure remaining only here and there.

It is to be noted that the number of strands is not uniform in the chromosome shown in Fig. 3. The drawing represents only one focal level, but the relative numbers of the striations are indicated fairly accurately. Careful study of all focal levels in the different regions indicates that in some regions, such as those marked *a* and *c*, the total number of striations ranges from 7 to 10. In contrast to this condition, the region at *b*, lying between *a* and *c*, shows a much larger number of striations. There appear to be more than 20 at this location, including all focal levels.

If the striations are assumed to represent preëxistent threads, this discrepancy might be interpreted as being due to breakage of some of the threads in such regions as *a* and *c*, or to previous additional division of the threads (chromonemata) in such regions as *b*, giving regions of the latter type a larger number. We have been unable, however, to find support for such an interpretation in studying the preparations themselves.

According to our observations, a thick region such as that shown at *b*, in a stretched chromosome, represents either a series of heavy chromatic discs which resist stretching, or else one of the " expanded " and disorganized regions often found in aceto-carmine preparations (see, e.g., Metz, 1935*b*, Fig. 5*A*, *B*, *C*). In the former case the resistance to stretching results in less distortion and in a retention of more of the " network " than in surrounding regions. Hence more striations are seen in such regions. In the other type, representing an " expanded " region, the original organization of the chromosome (in such a region) has been broken up by fixation and then the resulting structure has been further distorted by stretching. The fixation process results in the formation of an expanded region in which granules and vacuoles are intermingled, apparently at random. Not all preparations show this effect. At any one transverse level in such a region many vacuoles and granules are found. With moderate stretching a correspondingly large number of short striations appear. These apparently represent the boundaries of the vacuoles, and the large number is interpreted, not as an indication of the presence of many threads in the region, but as a reflection of the number of vacuoles, as just intimated.

When not subjected to mechanical distortion the expanded regions often show a more or less uniformly granular structure, without longitudinal striations. It is difficult to see how a structure of this type could thus appear if the chromosome originally contained numerous discrete longitudinal threads (chromonemata) unless these threads had become disorganized. If the latter assumption is made, however, it would carry with it the assumption that no threads would appear in such regions if they were stretched after fixation. But stretching does result in the apppearance of striations here, indistinguishable from those of other regions; and, as in the case of other regions, the direction of the striations coincides with the direction of stress.

The characteristics of these expanded regions are those to be expected on the present view, which considers the *fixed* chromosome as made up of continuous chromatic material, confined mainly to the discs, and discontinuous achromatic material in the form of droplets or vacuoles lying mainly between the discs. Expansion of the chromosome would be expected to give rise to a granular, vacuolated structure, without striations, but one in which striations could be produced by distortion just as in other regions. Furthermore, since the striations should, on this view, be numerically proportional to the number of droplets or vacuoles in a transverse section of the chromosome, there should be more of them in expanded than non-expanded regions. This agrees with the findings just considered and with those illustrated in Fig. 3.

Several cases have been examined in which an expanded region in one chromosome has adhered to the side of another chromosome and the two have then been pulled apart when the cell was crushed. In such cases that part of the expanded portion which is subjected to tension shows a realignment of materials to form striations, focusing at the point of adhesion. In some of these cases similar rearrangement of materials and focusing of the striations has been effected in the other

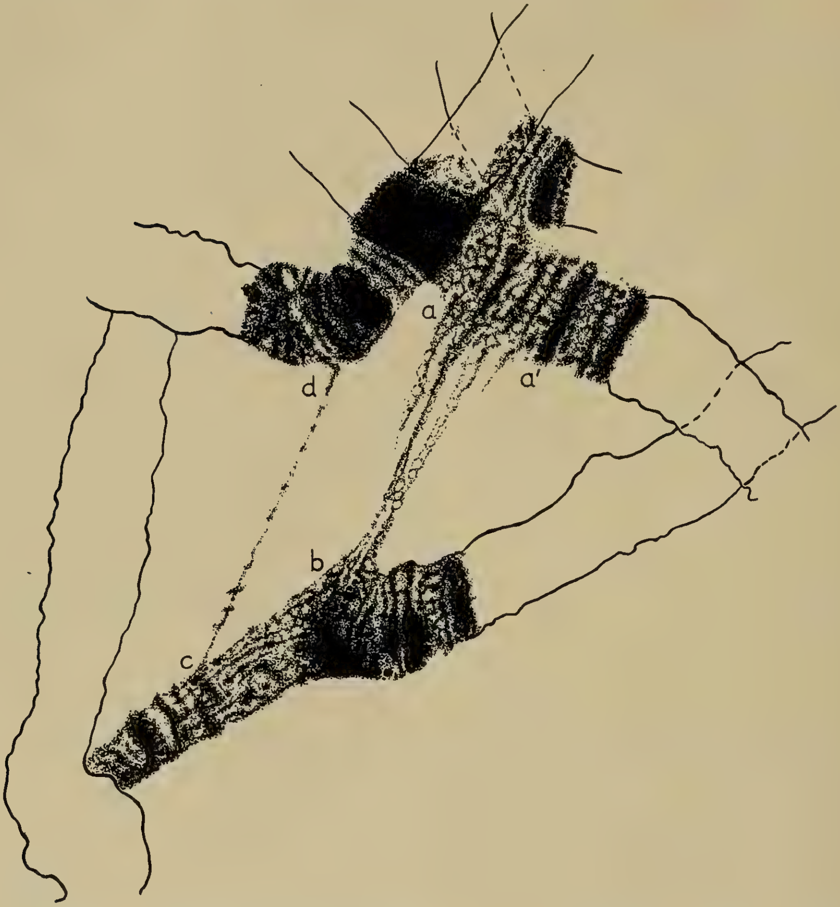


FIG. 4. Illustrating the distortion effect of lateral adhesion followed by mechanical separation of the two chromosomes. Chromatic materials from the bands between *a* and *a'* in one chromosome have been drawn out at right angles to the chromosome in the form of strands, connecting with similar strands from the other chromosome, at *b*. Such distortion should reveal the presence of longitudinal "threads" within the chromosome if detectable chromonemata are present; but none appear.

chromosome where no previous expansion had occurred. Between the two chromosomes extend strands of material similar to the striations. In these cases, as in the others just cited, it seems clear from the location and direction of the striations that they cannot represent pre-existent threads or chromonemata.

Fig. 4 provides further illustration of these features and also of the fact that the chromatic materials may be drawn out into threads extending at right angles from the chromosome. The two chromosomes represented at *a* and *b* in this figure had adhered laterally and were then pulled apart immediately after fixation. It is evident that materials from the chromatic discs between *a* and *a′* have been drawn out laterally in the form of strands and that these strands could not have been pre-existent as longitudinal threads in the original chromosome. It seems particularly significant also that there is no indication in such cases of bent longitudinal striations in the chromosomes, such as would be expected if the chromosomes were full of longitudinal threads before distortion.

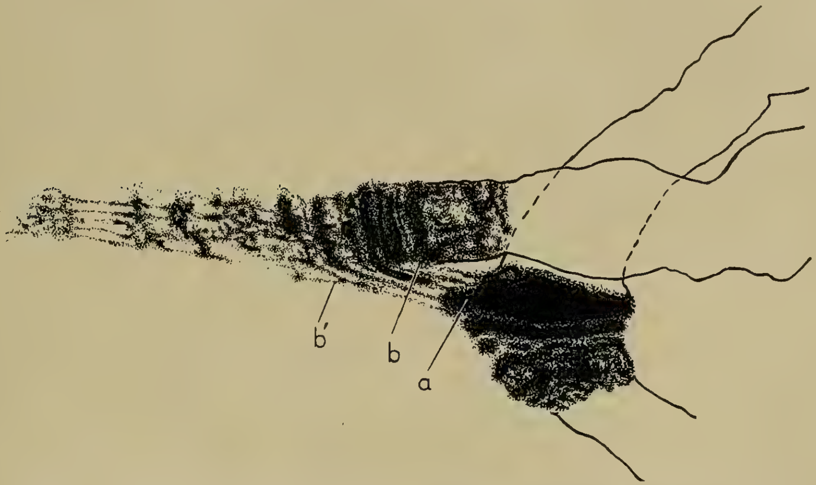


Fig. 5. Another example of lateral adhesion followed by mechanical separation of the two chromosomes. See text for description.

The resemblance of these lateral strands to longitudinal strands or striations within the stretched chromosome may be seen by comparison with the structures shown in the stretched region between *b* and *c*, in the lower chromosome. Another strand connecting two separate chromosomes is evident between *c* and *d*.

A particularly interesting example of lateral adhesion followed by stretching is represented in Fig. 5. Before the cell was crushed an adhesion had evidently formed between the thickened part of the lower chromosome at *a* and the region in the upper chromosome between the points marked *b* and *b′*. Then, in crushing the cell, the two chromosomes have been pulled somewhat apart in the region of adhesion due to a movement of the upper chromosome toward the left. Materials from the chromatic segments between *b* and *b′*, and perhaps to some extent also from segments in *a,* have been drawn out in the form of strands or threads. Chromosome *b* has been stretched toward the left, producing conspicuous longitudinal striations or strands which are indistinguishable in visible characteristics from the lateral strands connecting the two chromosomes. It seems especially significant that the lateral striations, which are obviously the products of distortion, not only resemble, but connect with, the longitudinal strands within the chromosome. Another feature of special interest to be noted here is the fact that the strands or striations do not extend strictly longitudinally in the upper chromosome but follow a somewhat diagonal path from *c* toward *a.* In other words, the lateral strands between the chromosomes, and those within the upper chromosome itself, coincide in such a way as to indicate clearly that they are both due to the tension between regions *a* and *c*. It is difficult to avoid the conclusion that these strands are all of the same type and are all artifacts produced by distortion.

DISCUSSION

The relation of the accompanying evidence to current hypotheses of chromosome structure will be considered in another paper. Brief mention of a few pertinent points, however, may be made at this time. It seems significant, not only that strands of chromosomal material which are evidently artifacts may resemble and connect with the " striations " within the stretched chromosome, but also that when strands are pulled out at right angles, as shown in Fig. 4, the distortion reveals no evidence of any longitudinal " threads " within the chromosome itself. If definite threads were present such distortion should bring them into view in the form of parallel *V*'s. Each longitudinal thread near the distorted edge should be pulled out somewhat, laterally, making a flattened *V*. We have numerous cases of the type represented in Fig. 4, in which materials from the discs have been pulled out at right angles, and none of them reveals any evidence of longitudinal thread-like structures inside the chromosome.

Such evidence tends to indicate not only that the " striations " are not chromonemata, but that no multiple chromonemata are present in the chromosome, unless such chromonemata are extremely small and delicate and are invisible in preparations thus far studied.

Our previous evidence has weighed strongly against the view that conspicuous chromonemata are present extending spirally around the periphery of the chromosome, as postulated by Koltzoff, and also against the view that conspicuous, essentially straight chromonemata fill the entire chromosome. It seemed possible, however, that a large number of chromonemata might be present if each were assumed to be independently and closely coiled. Such a structure might possibly give the appearance of a network or honeycomb such as we have seen in the fixed chromosomes; and when stretched it might give a striated appearance due to straightening out of the fine coils. If such threads were present, however, they should be revealed in cases such as those just considered; yet the preparations show no indication of anything of the kind.

Another feature, bearing indirectly on the above considerations, may also be mentioned at this point. In earlier papers (Metz, 1935*a, b*) it has been suggested that in the living chromosome, before shrinkage, the chromatic materials may be present in the form of continuous, relatively smooth discs, separated by intervening discs of achromatic material. Further comparative study of the effects of different fixatives, however (considered more in detail elsewhere), suggests the *possibility* that in the normal living chromosome no such sharp distinction exists between these two types of materials, and that the distinction in the shrunken chromosome is due to separation of components as a result of precipitation and coagulation. Such an interpretation, if substantiated, would indicate that the chromosome is composed of a series of different materials, representing the genes, but that they are not so sharply differentiated and isolated from one another as formerly supposed. Numerous serious difficulties stand in the way of such an interpretation, of course, and it is not desired to stress it on the basis of the present meager evidence. It seems worth while, however, to call attention to it and to its possible bearing on phenomena of crossing over and of " position effects " of different genes. If no sharp boundary lines existed between successive genes, crossing over would presumably be effected by the genes themselves, rather than by " inert " material between them; and the interaction of genes in close proximity to one another (" position effect ") would be subject to a different interpretation than that based on the current conception of distinctly separate units.

LITERATURE CITED

Bauer, H., 1935. Der Aufbau der Chromosomen aus den Speicheldrüsen von Chironomus Thummi Kiefer. *Zeitschr. Zellforsch.*, 23: 280.

Bridges, C. B., 1935. The structure of salivary chromosomes and the relation of the banding to the genes. *Am. Nat.,* **69:** 59.

Doyle, W. L., and C. W. Metz, 1935. Structure of the chromosomes in the salivary gland cells in Sciara (Diptera). *Biol. Bull.,* **69:** 126.

Koller, P. C., 1935. The internal mechanics of the chromosomes. IV. Pairing and coiling in the salivary gland nuclei of Drosophila. *Proc. Roy. Soc. B,* **118:** 371.

Koltzoff, N., 1934. The structure of the chromosomes in the salivary glands of Drosophila. *Science,* **80:** 312.

Metz, C. W., and E. H. Gay, 1934*a*. Chromosome structure in the salivary glands of Sciara. *Science,* **80:** 595.

Metz, C. W., and E. H. Gay, 1934*b*. Organization of salivary gland chromosomes in Sciara in relation to genes. *Proc. Nat. Acad. Sci.,* **20:** 617.

Metz, C. W., 1935*a*. Structure of the salivary gland chromosomes in Sciara. *Jour. Hered.,* **26:** 177.

Metz, C. W., 1935*b*. Internal structure of salivary gland chromosomes in Sciara. *Jour. Hered.,* **26:** 491.

Muller, H. J., and A. Prokofieva, 1935. The individual gene in relation to the chromomere and the chromosome. *Proc. Nat. Acad. Sci.,* **21:** 16.

THE RÔLE OF BLOOD CELLS IN EXCRETION IN ASCIDIANS

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How do ascidians dispose of the waste products resulting from purine metabolism? They have no tubular kidney comparable to the excretory organs present in many animals. The neural gland, the pyloric gland, and the terminal portions of the sperm ducts have been suspected of having an excretory function; but such suspicions rest on slight evidence, and are not generally credited. Some species have renal vesicles. When present, these are generally recognized as subserving a kidney function; but they are not present in all or even in most species of ascidians.

The renal vesicle in the Molgulidæ is a single large, closed, bean-shaped sac in which waste matter is gradually deposited and stored in the form of concretions throughout the life of the animal. In most members of the family Ascidiïdæ, and probably in some other families, there is, instead of one such kidney, a multitude of minute vesicles containing concretions. Structurally these concretions consist of concentric lamellæ and they may be simple or compound. Chemically they have been shown by means of the murexide test to contain uric acid (Kupffer, 1872, 1874; Lacaze-Duthiers, 1874). According to Dahlgrün (1901), who has studied the histology of these excretory organs, the vesicles have an epithelial wall one cell thick. A characteristic of the epithelial cells is the presence of an extensive system of fluid-filled vacuoles. Azéma (1926, 1928) has studied the structure and activity of isolated living vesicles of *Ascidiella aspersa* Mül. He finds that the cells forming the walls of the vesicles in this species always have a large vacuole near the nucleus and frequently young vacuoles in process of formation. In living material one may observe small concretions form upon the inner wall of the large vacuole, detach themselves, and float freely in it. By rupture of the main vacuole of a cell its concretions are expelled into the cavity of the multicellular renal vesicle. A small young vacuole then begins to increase and becomes substituted for the vacuole which ruptured.

In those ascidians that have no renal vesicle, and these are probably the greater number, the renal function appears to be taken care of by cells of the connective tissue and blood. Herdman (1888) mentioned the occurrence of excretory cells in *Botryllus,* and Roule (1884) reported cells of *Ciona intestinalis* that produced the characteristic reactions of uric acid and uric acid salts. They had thin walls and contained very small granules. Groups of these cells located in the connective tissue of the wall of the vas deferens he considered to be the kidney. Dahlgrün (1901) described the excretory organ of the Botryllidæ as consisting of a rather large number of isolated cells having an oval nucleus and containing bright brownish granules. He found these cells in the visceral region, especially in the space between the œsophagus and the stomach on one side and the rectum on the other side, usually immediately adjacent to the gut wall in the meshwork of stellate mesenchyme cells. In serial sections of *Ciona intestinalis* he found these cells with concretions in the visceral region only in the immediate neighborhood of the gut. They were present in small or large numbers depending upon the age of the animal.

More recently the findings of Azéma and my own observations show that these cells with granules of an excretory nature are blood cells and that they may circulate freely in the blood stream or be localized in the connective tissue.

In the course of comparative studies of ascidian blood I have observed blood cells with one or several vacuoles practically filling the cell body. Suspended in the fluid of the vacuoles Brownian granules are usually but not always present. In some species these granules are always colorless, as in *Perophora viridis;* in others they are colored. Some species may have cells with colorless granules and also cells of the same structure but of two or more color varieties, as is the case in *Clavelina picta.* Judged by the contents of the vacuoles, cells of this structure may be of at least two functional varieties: nutritive cells and excretory cells. In earlier papers (George, 1926, 1930*a*, 1930*b*) I described compartment and signet ring cells having granules of a fatty nature in some species. Cells with essentially similar structure may contain granules that are not of a fatty nature but apparently of purine composition.

Azéma (1928, 1929*a, b, c, d*), on the basis of various microchemical reactions, concludes that granules in vacuolated cells of the genus *Microcosmus* of the family Pyuridæ and in some species of the family Synoicidæ are composed of xanthine. The white pigment in certain cells of the Botryllidæ he calls a purine without specifying which one. In *Ascidia pellucida* he finds vesicular cells with concretions which present the distinctive characteristics of guanine. It is of some significance to note here that Sulima (1914) made a quantitative analysis of the purine content of the body tissues of *Cynthia microcosmus* (*Microcosmus vulgaris*). From his analyses he found a mean value of about 0.2 per cent uric acid and 0.3 per cent other purine bases (calculated as xanthine).

Within the living excretory cells the granules have certain characteristic optical qualities. By oblique or reflected light they shine in a dark background. This is a characteristic found in purine containing cells of other animals. Millot (1923), who made a detailed study of the guanophores of the lower vertebrates, found that crystals of guanine in the cells took on a brilliant aspect against a dark background. The reflecting granules in ascidian blood cells are one color by transmitted light and another color by reflected light. Such two-color granules, which may vary in size from the limits of visibility with the highest powers of the microscope up to concretions several microns in diameter, I have found in *Clavelina picta, C. oblonga, Distaplia bermudensis* of the family Polycitoridæ; in *Symplegma viride, Polycarpa obtecta,* and *Polyandrocarpa tincta* of the family Styelidæ; in *Pyura vittata* of the family Pyuridæ; in *Botryllus schlosseri* and *Botrylloides niger* of the family Botryllidæ. In *Ecteinascidia turbinata* Herdman and in *E. conklini minuta* Berrill of the family Ascidiidæ there are similar cells, but I could not see the intravacuolar granules in the living cells because of the presence of a film at the surface of the vacuoles. This film, like the granules in other species, was brown by transmitted light and white or yellowish white by reflected light. All of the above listed species have pigmented cells that are brown by transmitted light and white by reflected light. Some of them have in addition other two-color pigment cells. In *Distaplia bermudensis,* in addition to the typical brown and white cells, cells are present with granules that are purple by transmitted light and black by reflected light and also cells that are

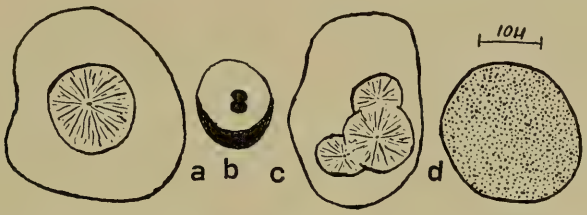

FIG. 1. Excretory cells of ascidian blood. *a*, *b*, and *c* are vesicular cells with reflecting concretions from the blood of *Polyandrocarpa tincta; b* is a young stage. *d* is a vesicular cell with reflecting granules from the blood of *Pyura vittata.*

yellowish brown by transmitted light and black by reflected light. Perhaps some impurity in the purine granules accounts for these variations.

Though commonly present in the form of granules (Fig. 1*d*), the reflecting pigments in ascidian blood cells may exist as simple or compound concretions (Figs. 1 *a, b, c*), as in *Polyandrocarpa tincta* and *Bolteniopsis prenanti.* The resemblance of these concretions to renal calculi is very striking. They are, in fact, microscopic intracellular calculi. They begin as tiny granules formed within cell vacuoles and grow by the addition of substance at the surface of these granules. Various stages in their development may be observed in living cells.

The vacuolated cells with purine granules and concretions seem to originate through differentiation of lymphoid cells and probably of fixed mesenchyme cells. Millot (1923) derives the guanophores of the lower vertebrates likewise from connective tissue cells and leucocytes of undetermined origin.

These excretory cells are present in the blood stream, but they need not remain in circulation. In *Ecteinascidia conklini minuta* I have observed them circulating in the blood spaces and also collected in clumps at the tips and near the bases of the siphon lobes, and collections of these cells cause flecks of white near the anterior end of *Clavelina oblonga* Herdman and here and there on colonies of *Botrylloides niger.*

Different species within the same family may have in one case multicellular renal vesicles, and in another, vesicular renal cells in the blood and connective tissue. Thus *Ascidia hygomiana* and *A. nigra,* according to my observations, and *A. mentula* and other species reported by Dahlgrün (1901) and other authors, have numerous multicellular renal vesicles with concretions, while *A. pellucida* is devoid of renal vesicles but has in the visceral region and in all the body wall pigment spots composed of vesicular cells containing concretions, which in this form present the distinctive characteristics of guanine (Azéma, 1929*d*). Also in the Pyuridæ Dahlgrün records renal vesicles for *Cynthia dura* and *Microcosmus serotus,* while Azéma (1929*a*) reports a number of species in which there are no renal vesicles but renal cells with purine concretions. I have been unable to find renal vesicles upon examination of serial sections of *Pyura vittata;* but renal cells are present. It seems not improbable, however, that renal vesicles and renal cells may be found in the same individuals of some species.

It seems evident from the foregoing facts that the ascidians make use of their blood cells in freeing the tissue fluids of the wastes of purine metabolism. Excretion granules have been found in free cells of other invertebrates, and even in the higher vertebrates it appears that blood cells participate in the excretory function. Bornstein and Griesbach

(1919) and Theis and Benedict (1921), upon examining many samples of blood, found that in some cases the quantity of uric acid was greater in the plasma and in others greater in the cells, and Benedict (1915) found that in ox blood uric acid is largely confined to the erythrocytes, though in chicken blood it is almost wholly in the plasma. The mode of its occurrence has been a matter of interest to biological chemists. Rose (1923), in his review of purine metabolism, calls attention to the fact that Bechold and Ziegler (1914), in order to explain the presence of more uric acid in the blood than the solubility of the lactim form accounts for, assumed, but did not demonstrate the presence of urates in colloidal condition. The demonstration of oxypurines in colloidal suspension in the vacuoles of ascidian blood cells gives some factual justification for Bechold and Ziegler's assumption.

There is another point in which the conditions in the ascidians may have some significance with regard to our conceptions of the nature of the excretory process in vertebrates. Partly because of its thinness and the absence of cytological evidence of secretory activity, there has been a tendency to look upon the epithelium of the renal corpuscle as being merely a filtration membrane. This assumption may be entirely correct; but the excretory activity of ascidian blood cells shows us that we are not justified in denying a secretory function to the glomerular epithelium merely because of its thinness. For in these cells we have a protoplasmic membrane so thin as to be merely a thin line under the highest magnification, and yet it appears to function as a glandular membrane, taking purine substances out of the blood plasma and storing them in the cavities of these unicellular bladders.

LITERATURE CITED

AZÉMA, MAURICE, 1926. Le mécanisme de l'excrétion chez les Ascidies. *Compt. Rend. Acad. Sci.,* **183**: 1299.

AZÉMA, MAURICE, 1928. Quelques aspects de l'excrétion chez les Ascidies. *Compt. Rend de l'Assoc. des Anat.,* 23 Reunion.

AZÉMA, MAURICE, 1929*a*. Notes sur les cellules excrétrices des Cynthiadæ. *Bull. Soc. zool. France,* **54**: 13.

AZÉMA, MAURICE, 1929*b*. Le tissu sanguin des Polyclinidæ. *Compt. Rend. Soc. Biol.* (Paris), **102**: 918.

AZÉMA, MAURICE, 1929*c*. Le sang des Botrylles. *Compt. Rend. Soc. Biol.,* **102**: 823.

AZÉMA, MAURICE, 1929*d*. Sur les cellules excrétrices d'Ascidia pellucida. *Bull. Soc. zool. France,* **54**: 617.

BECHOLD, H., AND J. ZIEGLER, 1914. Vorstudien über Gicht. III. *Biochem. Zeitschr.,* **64**: 471.

BENEDICT, S. R., 1915. Studies on uric acid metabolism. On the uric acid in ox and in chicken blood. *Jour. Biol. Chem.,* **20**: 633.

BORNSTEIN, A., AND W. GRIESBACH, 1919. Über das Verhalten der Harnsaüre im überlebenden Menschenblut. *Biochem. Zeitschr.,* **101**: 184.

DAHLGRÜN, WILHELM, 1901. Untersuchungen über den Bau der Excretionsorgane der Tunicaten. *Arch. f. mikr. Anat.,* **58**: 608.

GEORGE, W. C., 1926. The histology of the blood of Perophora viridis. *Jour. Morph.,* **41**: 311.

GEORGE, W. C., 1930*a*. The histology of the blood of some Bermuda ascidians. *Jour. Morph.,* **49**: 385.

GEORGE, W. C., 1930*b*. Further observations on ascidian blood. *Jour. Elisha Mitchell Sci. Soc.,* **45**: 239.

HERDMAN, W. A., 1888. Report on the Tunicata collected by *H.M.S. Challenger* during the years 1873–1876. Part. III. Report on the Scientific Results of the Voyage of *H.M.S. Challenger* during the years 1873–76. Zoology, vol. XXVII.

KUPFFER, C., 1872. Zur Entwicklung der einfachen Ascidien. *Arch. f. mikr. Anat.,* **8**: 358.

KUPFFER, C., 1874. Tunicata. Die zweite Deutsche Nordpolfahrt, Bd. II. Leipzig.

LACAZE-DUTHIERS, H. DE, 1874. Les Ascidies simples des côtes de France. *Arch. de zool. expér. et gén.,* **3**: 257.

MILLOT, JACQUES, 1923. Le pigment purique chez les Vertébrés inférieurs. *Bull. biol. France et Belg.,* **57**: 261.

PIZON, ANTOINE, 1899. Sur la coloration des Tuniciers et la mobilité de leurs granules pigmentaires. *Compt. Rend. Acad. Sci.,* **129**: 395.

ROSE, WILLIAM C., 1923. Purine metabolism. *Physiol. Rev.,* **3**: 544.

ROULE, L., 1884. Recherches sur les Ascidies simples des côtes de Provence (Phallusiadées). Ann. du Mus. Hist. Nat. Marseille, Zoologie T. 2, Memoire No. 1, pp. 1–270.

SULIMA, A., 1914. Beiträge zur Kenntnis der Harnsäurestoffwechsels niederer Tiere. *Zeitschr. f. Biol.,* **63**: 223.

THEIS, R. C., AND S. R. BENEDICT, 1921. Distribution of uric acid in the blood. *Jour. Lab. and Clin. Med.,* **6**: 680.

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CONTENTS

Page

THIRTY-EIGHTH REPORT OF THE MARINE BIOLOGICAL LABORATORY 1

WATERMAN, A. J.
The Membranes and Germinal Vesicle of the Egg of Sabellaria vulgaris 46

TYLER, ALBERT
On the Energetics of Differentiation, III 59

TYLER, ALBERT
On the Energetics of Differentiation, IV 82

HARVEY, ETHEL BROWNE
Parthenogenetic Merogony or Cleavage without Nuclei in Arbacia punctulata 101

COE, W. R.
Sequence of Functional Sexual Phases in Teredo 122

RAKESTRAW, NORRIS W.
The Occurrence and Significance of Nitrite in the Sea 133

FISH, CHARLES J.
The Biology of Oithona similis in the Gulf of Maine and Bay of Fundy 168

BEAMS, H. W., and R. L. KING
The Effect of Ultracentrifuging upon Chick Embryonic Cells, with special reference to the "Resting" Nucleus and the Mitotic Spindle 188

DELAMATER, ARLENE JOHNSON
The Effects of Heavy Water upon the Fission Rate and the Life Cycle of the Ciliate, Uroleptus mobilis 199

YOUNG, ROBERT T., AND DENIS L. FOX
The Structure and Function of the Gut in Surf Perches (Embiotocidae) with reference to their Carotenoid Metabolism 217

METZ, C. W.
Effects of Mechanical Distortion on the Structure of Salivary Gland Chromosomes 238

GEORGE, W. C.
The Rôle of Blood Cells in Excretion in Ascidians 249

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THE REACTIVATION BY CUTTING OF SEVERED MELANOPHORE NERVES IN THE DOG-FISH MUSTELUS

G. H. PARKER

(*From the Woods Hole Oceanographic Institution, Woods Hole, Mass.*[1])

When the melanophore nerves in many lower vertebrates are cut, the areas of skin thus denervated quickly turn dark and remain so for some considerable time. This darkening results from a dispersion of pigment in the melanophores of the denervated areas. Such occurrences were apparently first noted in the common chameleon in 1852 by Brücke, whose observations were confirmed on South African species by Hogben and Mirvish (1928) and by Zoond and Eyre (1934). In bony fishes similar darkenings of the skin as a result of nerve cutting have been recorded by Pouchet (1876), von Frisch (1911), Wyman (1924), Hewer (1927), Giersberg (1930), and Smith (1931), to mention only a few of the more recent workers. Brücke expressed the opinion that melanophores were related to their nerves as muscle-fibers are to motor nerves and that, since the cutting of motor nerves induces paralysis in the neuro-muscular system, the same should be true of melanophore nerves and their effectors. He therefore advanced the idea that severed melanophore nerves with their expanded color-cells are in a state of paralysis. This interpretation of the condition of these parts has been accepted by almost all investigators in this field, even the most recent (Keller, 1895; von Frisch, 1912*a*; Spaeth, 1916; Giersberg, 1930; Zoond and Eyre, 1934; Sand, 1935). In opposition to this view I have advanced the idea that when a melanophore nerve is cut the injury thus inflicted throws the nerve into a state of unusual but temporary activity which may last, however, even for some days. This opinion is based chiefly upon observations on the caudal bands of the common killifish, *Fundulus heteroclitus* (Parker, 1934).

If a dark band is produced by the usual cut in the tail of a pale *Fundulus,* this band, as is well known, will fade out in the course of a few

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[1] Contribution No. 113.

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days and become almost if not quite indistinguishable in tint from that of the rest of the fish. If now a second cut is made slightly distal to the first one and within the limits of the original band, a second somewhat smaller band will form between the new cut and the edge of the fin. This second band may be induced at any period after the first band has faded sufficiently and before the degeneration of the melanophore nerves has set in, a disturbance which begins to appear some five days after the initial cut has been made. Any time during these first five days on application of a cold block to such a dark band the part of the band distal to the block may be temporarily obliterated. Or if a cut is made proximal to a block, in a tail in which there is no band, a band will form from the cut to the block but not beyond it, though a cut distal to the block will induce a second band from the new cut to the edge of the tail. These several conditions show that the dark band that immediately results from a cut in the tail of *Fundulus* is not due to the paralysis of the detached portion of the neuro-melanophore system as maintained by almost all previous workers (paralysis hypothesis) nor to the blocking of impulses as suggested by Zoond and Eyre (1934), but is occasioned by the local irritation of the dispersing melanophore nerve-fibers, an irritation that may last for some days (superactivity hypothesis).

The situation thus briefly outlined is based upon experiments carried out on *Fundulus*. During the past summer an opportunity was offered at the Woods Hole Oceanographic Institution to apply similar tests to elasmobranchs. The smooth dogfish, *Mustelus canis,* possesses only one set of melanophore nerves, namely, those concerned with pigment concentration. Consequently when a cut is made in the pectoral fin of this fish, a pale band appears in this organ (Parker and Porter, 1934). Such bands disappear in the course of time as the dark bands in *Fundulus* do. If after such a band in a smooth dogfish has faded out (Fig. 1), a second

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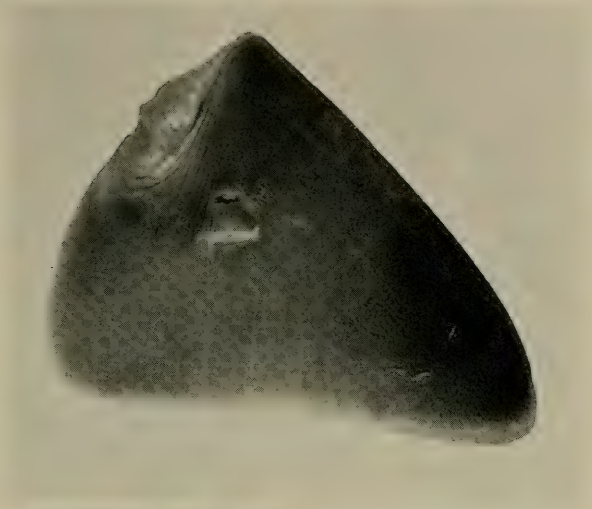
EXPLANATION OF FIGURES

FIG. 1. Right pectoral fin of *Mustelus* seen from above. The initial cut (lower) was followed by the formation of a pale band extending from the cut to the edge of the fin. After this band had darkened as appears in the photograph a second cut was made (upper) proximal to the first one. This second cut produced no observable paleness in the fin.

FIG. 2. Left pectoral fin of *Mustelus* seen from above. The initial cut (upper) induced the formation of a pale band extending from the cut to the edge of the fin. After this band had darkened (Fig. 1), a second cut (lower) was made distal to the first one. This second cut produced the faint splotchy pale area which extends from the region of the cut to the edge of the fin.

FIG. 3. Right pectoral fin of *Mustelus* seen from above. The pale band from the initial cut had darkened before the second cut distal to the first one was made. This second cut excited the formation of a very distinct distal splotch within the area of the former band.



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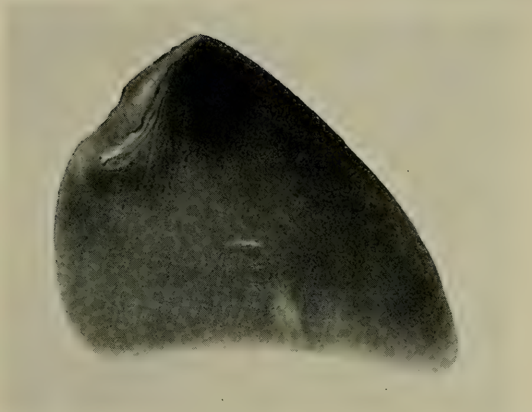

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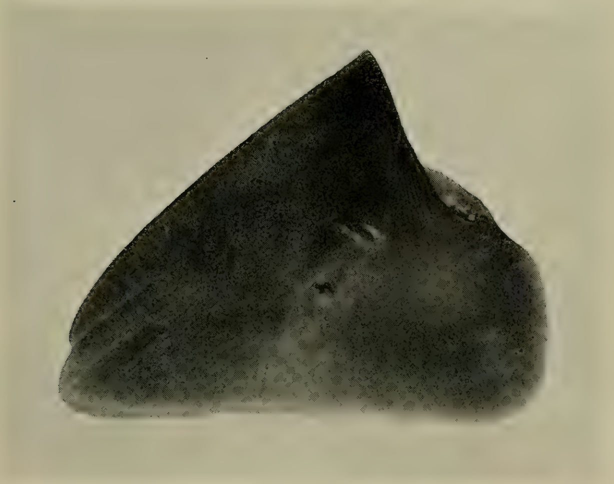

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FIGS. 1–3

cut is made within the limits of the first one and distal to it, a second fainter band commonly much limited in extent will become visible within the area of the first (Figs. 2 and 3). In *Mustelus* both the primary and the secondary bands are much less regular than in *Fundulus.* This is probably due to the less regular course of the melanophore nerves in the dogfish than in the killifish and to the fact that in the dogfish the one nervous reaction, concentration, is overcome by a humoral response, dispersion, dependent upon a diffuse pituitary neurohumor in the blood. The important point in this discussion, however, is the fact that in *Mustelus* a pale band after it has become dark can be revived by the recutting of its nerves showing that these nerves were not paralyzed by the first cut but remained functional and that the blanching of the band is the result of some form of irritation in the cut and not of paralysis nor of the blocking of impulses. These observations then are in accord with those already reported for *Fundulus* and in this respect support the superactivity hypothesis. Attempts were also made to produce bands in the pectoral fins of the skate *Raja erinacea,* which shows well marked color-changes (Parker, 1933), but the cutting of nerves in the pectoral fins of this fish was not followed by the formation of bands to any observable extent. In this respect *Raja erinacea* is like *Squalus acanthias* in which fin bands are scarcely if at all elicitable (Parker, 1936).

REFERENCES SUBSEQUENT TO 1929

GIERSBERG, H., 1930. Der Farbwechsel der Fische. *Zeitschr. vergl. Physiol.,* **13**: 258–279.

PARKER, G. H., 1933. The color changes of elasmobranch fishes. *Proc. Nat. Acad. Sci. Washington,* **19**: 1038–1039.

PARKER, G. H., 1934. The prolonged activity of momentarily stimulated nerves. *Proc. Nat. Acad. Sci. Washington,* **20**: 306–310.

PARKER, G. H., 1936. Color changes in elasmobranchs. *Proc. Nat. Acad. Sci. Washington,* **22**: 55–60.

PARKER, G. H., AND H. PORTER, 1934. The control of the dermal melanophores in elasmobranch fishes. *Biol. Bull.,* **66**: 30–37.

SAND, A., 1935. The comparative physiology of colour responses in reptiles and fishes. *Biol. Rev.,* **10**: 361–382.

SMITH, D. C., 1931. The influence of humoral factors upon the melanophores of fishes, especially Phoxinus. *Zeitschr. vergl. Physiol.,* **15**: 613–636.

ZOOND, A., AND J. EYRE, 1934. Studies in reptilian colour response. I. The bionomics and physiology of the pigmentary activity of the chameleon. *Phil. Trans. Roy. Soc. London, B,* **223**: 27–55.

PHYSIOLOGY OF THE MELANOPHORE SYSTEM IN THE CATFISH, AMEIURUS [1]

A. A. ABRAMOWITZ

(From the Biological Laboratories, Harvard University, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

I. Introduction

The color changes of the catfish, *Ameiurus nebulosus* (Leseur), have been studied recently by Parker (1934*a*) in relation to neurohumors. This fish can be made to assume a yellowish-green color in an illuminated white vessel within three hours, and becomes remarkably dark in an illuminated black vessel within one hour. Such changes are known to be due to the concentration or dispersion of the pigment contained within pigment cells or chromatophores, and are brought about not only through stimulation of chromatophore nerves but also through the coöperation of hormones. One of these hormones is that of the pituitary gland which, according to Parker, is secondary to the dominant nervous system. Its effect on the melanophore pigment of the catfish is dispersion, an effect characteristic of intermedin, the hormone of the intermediate pituitary (Zondek, 1935). Another hormone which this paper will show to be in operation in special states is similar in effect to adrenalin. The existence of such a substance has already been advocated by Smith (1931) to be responsible for the pallor of denervated regions of melanophores in *Phoxinus*. In the year following Smith's publication, however, a rather novel hypothesis (the neurohumoral theory, Parker, 1932) was advanced to explain the behavior of denervated melanophores. Thus, two mechanisms have been advanced to account for the reactivity of denervated chromatophores. It was therefore decided to reinvestigate the catfish from both of these points of view, and to determine the factors and mechanisms which would account for all of the demonstrable chromatic responses of this fish.

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[1] This work was carried on under the direction of Professor G. H. Parker and, with the exception of the section on the pituitary gland, was submitted as part of a thesis presented for the doctorate degree, Harvard University, 1935. To Professor Parker I am greatly indebted for much advice and innumerable kindnesses. Acknowledgments for research facilities are due Dr. M. H. Jacobs, Director of the Marine Biological Laboratory, Woods Hole, where these investigations were carried on in the summers of 1933 and 1934.

II. MATERIAL AND METHODS

The catfish, *Ameiurus,* was used for the experiments here being reported. Specimens were obtained in Cambridge during winter months from local dealers, and during summer months from neighboring fresh water ponds in the vicinity of the Marine Biological Laboratory, Woods Hole. In the laboratory they were kept in large tanks of running water. When used for experimental purposes the animals were maintained in white porcelain dishes or in large glass vessels the outsides of which had been painted black. Such vessels have been used for many years in this laboratory for the induction of desired chromatic responses. Ordinary Mazda lamps provided illumination of the white and black backgrounds, both being illuminated with lamps of equal light intensities. Photographic darkrooms were employed in the study of the behavior of animals to light or darkness, and, unless otherwise stated, all experiments were carried out at room temperature.

III. EXPERIMENTS

A. The Relation of Melanophores to the Nervous System

1. *Pigment Motor Pathways.*—The dominance of the nervous system over the pigmentary responses of teleosts has never been threatened seriously by any other agency. The general arrangement of the pigmentary nerve tracts has been determined for many fishes, but for practical reasons this was omitted in the catfish. It is probable that the catfish is not different in this respect from *Phoxinus* and other European fishes (von Frisch, 1911), the English dab (Hewer, 1926*b*), the scorpion fish (Smith and Smith, 1934), and *Fundulus* (Spaeth, 1913; Abramowitz).[2] It may be stated with a good measure of accuracy that the general arrangement of the pigmentary nervous tracts is more or less constant among teleosts. Pigment motor fibers extend from the brain for some distance through the spinal cord. At a definite point, which may vary slightly for different fishes, the fibers leave the spinal cord and enter the sympathetic chains from whence they are distributed to all portions of the integument mainly by way of the segmental spinal nerves. When any part of this system is disrupted, a darkening of the skin to which the cut nerves are distributed is apparent. There seems to be a no more universal response of teleost melanophores than that of expansion following a denervating operation. The use of denervating operations in the elucidation of normal pigmentary responses is, as Parker's work has shown, well worth while, for denervated cells may be studied side by side with normal cells in a small area of the integument.

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[2] Unpublished work: "The Physiology of the Innervation of Fish Melanophores," doctorate thesis, Harvard University.

The most convenient region in the body of the catfish in which the denervating operations may be performed is the caudal fin. This fin is supported by some fifteen rays. Parker (1934*a*) demonstrated that the main nerves pass out in the rays to the chromatophores of the tail and consequently the cutting of a ray by means of a transverse incision severs the nerve which controls that part of the fin. A dark band extending from the incision to the distal edge of the fin is therefore produced.

2. *Blinding.*—The melanophores in these dark bands (primary caudal bands) are, of course, denervated and therefore incapable of ordinary responses to backgrounds, responses which are mediated by the eyes. That removal of the eyes causes a darkening of the animal is an observation reported repeatedly not only by investigators interested in its chromatic responses but also by those having occasion to use this fish for other purposes. Optic enucleation is much like cutting a peripheral nerve, the main difference superficially being that blinding results in a general effect, whereas destruction of a peripheral nerve produces only a local response. Blinded catfishes naturally lose the capacity to respond to backgrounds, but they do not lose all of their chromatic responses, for they react in a very positive manner to variations of light intensities. This matter, however, will be taken up in a subsequent section.

3. *Denervated Melanophore Responses.*—Just as the melanophores of blinded animals do not lose their capacity to react, the denervated cells of the caudal bands do not suffer loss of the ability to undergo pigment alterations. The early investigators spoke of denervated cells as paralyzed, but Parker has amply demonstrated complete activity of these so-called paralyzed cells. The following account is taken from Parker (1934*a*) and serves to illustrate what has been designated as neurohumoral activity. A transverse cut across one or two fin rays, as has been mentioned, produces a dark band in which the pigment of the melanophores is fully dispersed. This dark band fades if the fish is kept in an illuminated white vessel for three days but suffers no change in tint if the animal is maintained in a black vessel. If the animal which has been kept in a white vessel and whose caudal band in consequence of this treatment has become fully pale is transferred to a black dish, the caudal band darkens at the same time as or slightly later than the remainder of the animal. The fading of a dark band is accomplished, according to Parker, by a concentrating neurohumor which diffuses into the denervated area as a secretion from the intact concentrating nerve terminals of the flanking tail areas. The reverse change is due to the combined effects of the pituitary hormone and of a dispersing neurohumor secreted from the intact dispersing nerve terminals of the adjacent areas. Of these two dispersing agencies, the pituitary hormone is the weaker,

as indicated by the fact that the same neurohumoral responses occur in hypophysectomized catfishes (Parker, 1934*a*).

4. *Degeneration and Regeneration of Radial Nerves.*—As is expected, the melanophores of the caudal band remain functionless as far as ordinary nervous responses are concerned. The neurohumoral experiments of Parker bear evidence to this. Moreover, if a totally dark animal possessing a dark caudal band is stimulated electrically through the roof of the mouth, the animal pales within several minutes but the band remains fully dark at the end of this time. In a similar but totally pale animal an incision made in the tail tissues somewhat anterior to the point of initial transection causes only a dark block between the two transverse cuts. These standard experiments demonstrate that no central impulses can be transmitted across the cut nerves. Following the initial incision used to produce primary bands, the cut nerves undergo progressive degeneration (Parker, 1934*a*) so that thirteen days later their distal tracts have degenerated completely.

TABLE I

Rate of regeneration of concentrating radial nerves of Ameiurus nebulosus *in millimeters per day.* This table is a sample table of data obtained in the study of chromatophore nerve regeneration. These determinations were made during the months of July and August, and consequently the rate is slightly higher than that which has been given in the text. The average rate computed at various intervals of the year is 0.51 mm. per day.

Length of denervated area	Rate of regeneration in millimeters per day measured at the end of the following days:							
	4.3	9	12	17	22	24.3	30	Average
Fish I........ 16 mm.	0.70		0.50	0.53	0.55		0.43	0.54
Fish II....... 16 mm.	1.15	0.95	0.92	0.75		0.53	0.53	0.80
Fish III...... 14½ mm.	1.0	0.78			0.50		—	0.76
Fish IV...... 17½ mm.	0.93	0.89		0.59	0.55		0.47	0.72
Fish V....... 16½ mm.	0.70		0.50	0.41	0.45	0.54	—	0.52

— = regeneration completed. Average 0.67 mm. per day.

Depending on the temperature and possibly other factors, regeneration of chromatophore nerves begins about two weeks after the initial operation and commences from the central stumps of the cut nerves. Nerve regeneration progresses at the rate of a half-millimeter a day (Table I), as determined by the method described by Abramowitz (1935). Active regenerative growth of nerves, as reflected by the reactivity of melanophores, may be demonstrated by a variety of methods.

If a totally dark animal (operated on three weeks previously) is placed on a white background or stimulated electrically, the typical pallor results and this pallor includes a portion of the originally denervated area. The reactive area of the band is therefore a reflection of the extent of the regenerated fibers. The portion of the band which has not been invaded by regenerative growth remains fully dark. The pale, or reactive area of the band increases daily at the expense of the dark, or unreactive area, and the process of nerve regeneration may be studied in the same animal, day by day, simply by repeating the above described procedure. The same phenomenon may be demonstrated in the reverse manner. Several animals in which nerve regeneration has begun at approximately the same time are made totally pale. Secondary incisions made anterior to the original transections are introduced at three-day intervals. The lengths of the dark streaks thus formed in the originally denervated band are measured distal to the initial incisions and the rate calculated. This average, and several other mean rates determined by still different methods, is a half-millimeter per day. Eventually nerves regenerate through the entire caudal band. This is complete within a month and at the end of this time all of the caudal band melanophores respond in a fashion similar to that of the unoperated portions of the tail (Figs. 1–6).

While nerve regeneration is progressing, however, the condition of the caudal band is such that part of it is normally innervated and therefore functional, and the remainder totally denervated (Fig. 7). Such preparations have been used in most of the experiments and will be referred to as "regenerating specimens." For convenience, that part of the band which has been innervated by regeneration will be called the "regenerated portion," and that part which has not yet received regenerated nerves, "the unregenerated portion."

B. *Reactions of Melanophores in Blinded Animals*

It was previously mentioned that blinded catfishes do not lose the capacity to undergo changes in shade. The mechanism by which such responses are brought about is not well known but is of considerable interest. Is the reactivity of the melanophores in blinded animals the result of the direct stimulation by light? This question is one of long standing, and no universal solution to it has been given. In the catfish, however, the following experiments show that the activity of melanophores in blinded animals is not a direct effect of light but is the result of a process which involves the transmission of impulses over the nervous system.

1. *Reactions of Innervated and Denervated Melanophores.*

a. Normal animals on white or black background and in darkness.—For purposes of comparison, a series of animals was tested after Parker's method. One-, two-, and three-ray transections were made in six white-adapted animals, and the animals subsequent to this treatment were maintained on a white background. The ultimate fading of the bands was completed after the following intervals:

Set 1 (1-ray transection)	2 days
Set 2 (2-ray transection)	5 days
Set 3 (3-ray transection)	10 days

Similar sets of animals maintained on a black background showed no changes after ten days. Another series placed in total darkness showed responses which were not uniform and perhaps deserve more detailed description. The catfish when kept in total darkness was reported by Parker (1934*a*) to be darker than intermediate controls. In another paper (Parker, Brown, and Odiorne, 1935) the animal was described as

EXPLANATION OF PLATE I

All photographs are from life and of natural size. FIGS. 1–6 are photographs of the tail of the same catfish during regeneration of nerves to the caudal band.

FIG. 1. Tail of a white-adapted catfish one hour after a transverse incision was made across two rays.

FIG. 2. Same a week later.

FIG. 3. Same eighteen days after the incision was made.

FIG. 4. Same thirty-eight days after making the incision. Regeneration of nerves to the band had progressed eight millimeters as evidenced by the formation of a light band distal to the incision when the animal was placed on a white background for an hour. The dark region is the unregenerated portion of the band.

FIG. 5. Same forty-two days after the incision was made.

FIG. 6. Same forty-six days after the incision was made. Regeneration of nerves is almost complete.

FIG. 7. Tail of a light catfish in which regeneration of nerves to the caudal band was half completed. The dark portion is the unregenerated portion of the band.

FIG. 8. Tail of a blinded catfish kept in the dark room. Regeneration of nerves to the caudal band is three-quarters completed. The unregenerated portion of the band remains dark but becomes progressively pale as regeneration of nerves proceeds to the denervated melanophore.

FIG. 9. Tail of regenerated preparation treated with adrenalin (0.2 cc.). The photograph was taken three hours after the injection was made, during which time the animal was kept in black surroundings. The innervated areas darken, but the denervated (unregenerated portion) area remains pale for several hours longer.

FIGS. 10–12 are photographs of the tail of the same catfish.

FIG. 10. Tail of a regenerated preparation during electrical stimulation of the roof of the mouth for two minutes.

FIG. 11. Same five minutes after cessation of stimulation.

FIG. 12. Same ten minutes after cessation of stimulation. The unregenerated portion of the tail remains pale several hours longer.

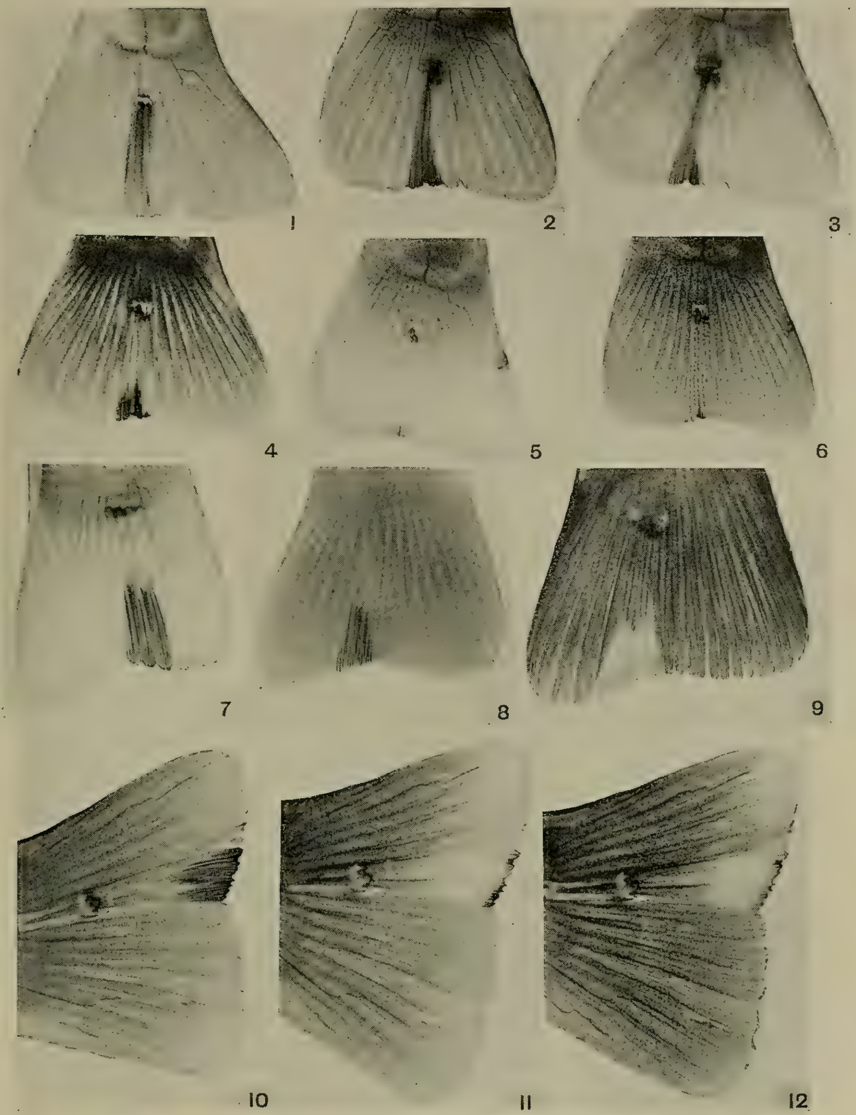
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Plate I

pale when kept under the same conditions. I have found no uniformity concerning this situation. Some animals kept in the darkroom were pale, though not as pale as blinded animals kept in the same room nor as pale as normal fishes kept in illuminated white vessels. The condition of the caudal bands in these animals was likewise variable. The bands were usually dark, but occasionally they were completely pale. Furthermore, bands which were pale one day were found to be fully dark on the following day, and vice versa. Observations were repeated many times during the course of a year in an attempt to obtain more uniform data, and while the tint of the animal as a whole was always close to an intermediate condition, that of the bands was extremely variable.

Since the results were not uniform, closer attention was paid to the condition of the animals before they were placed in total darkness. Hence animals in the two extreme states of background adaptation—very pale and extremely dark, with corresponding caudal bands—were placed in the darkroom for various periods of time. The following protocol is typical for a series of twelve animals.

Protocol I

1933

December 25—10 A.M. Two fishes, one light-adapted for ten days with its induced caudal band faded, and the other very dark with its band equally as dark, were placed in the darkroom.

December 26—11 P.M. Both animals equally dark in tint, more to the dark side of the midpoint. Both caudal bands now darker than the rest of the animal.

December 28—7 P.M. Both animals light—more to the light side of the midpoint. Bands still deeply dark. (One of the animals became very excited during the observation, whereupon it paled markedly.)

December 29—11 A.M. Both fishes darker than previous observation, about equal in tint. Bands still darker than body tint. (One animal excited.)

December 30—1 P.M. Both equally dark. One band completely blanched in the animal which had been excited on the previous day; the other band as before.

January 1—10 A.M. One fish lighter than the other. Both bands darker than the general tint. (One fish excited again by the approach of the observer.)

January 3—11 A.M. One fish slightly lighter than the other, both about intermediate. One band dark, the other, in the fish which was previously excited, completely pale.

Fishes which were of intermediate tints and which exhibited fully dark bands when placed in darkness remained in much the same condition for eleven days except when disturbed by the approach of the observer.

These results indicate that regardless of the initial color (pale, intermediate, or black), the animals when placed in total darkness come to a state intermediate between the two extremes. This intermediate condition is not a constant one and the animals may be slightly paler or darker than that which one may arbitrarily term an intermediate control. The fate of the bands is not consistent and depends in great measure upon the excitability of the animals. It is significant that a totally pale band will become fully dark when a pale animal is placed in total darkness. This takes place without any obvious stimulation and is interpreted as a capacity of denervated cells to expand in the absence of concentrating influences initiated by the eyes. Both observations—the behavior of denervated bands in excited animals and the darkening of pale bands in undisturbed specimens kept in the darkroom—will receive detailed treatment in the following pages.

b. Blinded animals on white or black background, and in darkness.—Blinded specimens, as Parker states, cannot respond to background influences. They therefore remained quite dark on either illuminated white or black backgrounds, and their caudal bands were equally dark. Blinded animals placed in darkness showed interesting responses. In all cases the fish as a whole was quite pale but the band was extremely dark. Such a condition persisted for twelve days at the end of which time regeneration of nerves into the denervated band commenced. As regeneration proceeded the dark band became progressively pale so that at the end of a month the entire tail was uniformly pale. These observations show that the pallor of blinded catfishes kept in darkness is a response mediated by the nervous system (Fig. 8).

Having shown that activity of melanophores in blinded animals in total darkness is dependent upon the nervous system in contrast to direct activity of the cells themselves, it became necessary to demonstrate that the effect of light was of a similar nature. When regenerating specimens of blinded catfishes kept in darkness were illuminated they became black within a minute or two. This reaction included the pale regenerated portion of the band. The unregenerated portion of the band, of course, suffered no change inasmuch as it was dark before the animal was illuminated. Such an experiment does not discriminate between the effects of light upon normal and denervated cells. If preparations could be made of blinded catfishes whose tails contained a band, the unregenerated portion of which was pale, the differential response of the innervated and denervated melanophores of the band would at once determine whether or not the effect of light is a nervous phenomenon. I have been unable to prepare blinded animals in this fashion, and consequently resorted to the use of freshly denervated bands. Several totally pale animals were quickly blinded and then returned to illuminated white vessels. Within thirty minutes, the animals became extremely dark but their denervated tail sectors remained totally pale. One hour later the bands became noticeably gray, and by the next day they were as dark as the remainder of the integument. This condition persisted as long as they were illuminated, and the band showed no further changes. The response of pale blinded animals to light, like the reverse reaction, is carried out through nervous channels. Both sets of experiments confirm von Frisch's statement (1911), that the effect of light on the melanophores of most fishes is indirect.

2. *The Dispersed Condition of Denervated Melanophores.*—In the foregoing account a seemingly inscrutable situation has been disclosed. Animals when blinded and transferred to a darkroom attain a condition in which the denervated band is fully dark and the remainder of the animal pale. In the light of Parker's neurohumoral work, one would not expect such a condition to exist for more than two or more days; nevertheless, the band remains dark until nerve regeneration sets in some two weeks after the caudal bands were induced. The reason that denervated cells are fully and indefinitely expanded under such circumstances is not obvious unless it be that the natural tendency of a denervated chromatophore is expansion, and that this particular situation happens to be one which permits such an intrinsic property of a denervated pigment cell to assert itself. This explanation, admittedly, appears to constrain a compromise of an otherwise paradoxical situation; none the less, the following data fit such an explanation:

a. Normal animals.—In black-adapted animals the denervated band always appears as dark as the remainder of the animal. In any intermediate state the band is always darker than the general tint of the animal. In such animals of intermediate tints the greater expansion of the band melanophores cannot be due to the already-known dispersing or expanding agencies for if such were the case the band could not theoretically be darker than the remainder of the fish. Furthermore, the condition of the cells in the caudal sector cannot be an effect of the prolonged activity of severed nerves (Parker, 1934*b*), for the same condition prevails after these nerves must have degenerated completely. Finally, tail bands are darker than innervated regions of skin in animals maintained in the darkroom, except in special cases where it will be shown that the exception is the result of a different mechanism.

b. Blinded animals.—Here the evidence is more direct. Several animals kept on a white background for ten days were quickly blinded and placed immediately in total darkness. Their caudal sectors were, of course, fully pale in consequence of the prolonged white-background adaptation. Twenty-four hours later, these animals were found to be as pale as normal controls which had been white-adapted for ten days, yet their bands were extremely dark and remained in this state for two weeks until nerve regeneration proceeded to the band melanophores.

The full expansion of denervated melanophores occurs without any apparent stimulation, and seems to be an intrinsic property manifested only when such denervated cells are free of concentrating environment induced by the eyes.

3. *The Rôle of the Pituitary Gland.*—The experiments described in the preceding pages were performed on normal animals modified in particular aspects, and the results were presented as more or less conclusive of certain problems in the physiology of fish melanophores. In the final analysis, however, the results are not to be regarded as absolute because another melanin-dispersing agency, the pituitary gland, has not been taken into consideration. A series of experiments was therefore designed toward an analysis of the rôle of this gland in the chromatic responses of the catfish. Parker has attributed a minor rôle to the pituitary gland, inasmuch as neurohumoral responses occur in animals without pituitaries. It seemed desirable to repeat and extend Parker's observations on hypophysectomized animals, and to determine what influence the pituitary may have on the reactions recorded in the previous pages. About sixty hypophysectomized animals constituted the experimental material, and the results are in accord with what Parker has already established.

Twenty animals were maintained on white dishes until all were uniformly pale. Ten were hypophysectomized and returned to their white dishes. Three hours later denervated caudal bands (two-ray transections) were induced into all of the animals, after which they were left undisturbed. At the end of from five to six days the denervated bands faded in both the normal white-adapted and the hypophysectomized white-adapted animals. There was some individual variation, it is true, but there was no marked difference in the time of the end response of the denervated cells of both sets of animals.

Twenty animals were kept in black dishes for several days, and sixteen of these were hypophysectomized and returned to their original dishes. One hour later denervated bands equal to those of the white-adapted series were introduced, and the animals left undisturbed for six days. At the end of this time the four black-adapted normal animals, including their denervated bands, were entirely black. The hypophysectomized black-adapted animals were dark, though not black, and their bands were as dark or slightly darker than the remainder of the integument. Many animals exhibited a completely blanched tail sector, but this condition can be attributed to the mechanism described in the final section.

The environments of these two series of experimental animals were reversed, the white-adapted series was placed on black background, and the black-adapted, on white. The white-adapted normal animals darkened within three hours; this response being shared by the denervated band. The white-adapted hypophysectomized animals darkened within three hours but the denervated band remained fully pale for several days, after which they assumed a moderate degree of melanophore expansion. The black-adapted normal specimens when transferred to the white background paled at the end of thirty-five hours; the band paled at the end of two and a half days. The black-adapted hypophysectomized animals lightened from one-half to eight hours, and their caudal bands bleached after a day. The pituitary gland is not indispensable for the color changes of this fish, yet it would seem from the above results that it acts as a " lubricant " for the machinery of melanin dispersion (especially for denervated cells) and as a counter-weight which the concentrating chromatophore nerves must overbalance to bring about melanophore contraction.

The previous work on blinded specimens showed that their reactions were under nervous control. It is conceivable, however, that light may stimulate photoreceptors of the skin which in turn would reflexly excite the pituitary to darken the animal and to maintain this dark coloration as long as the animal was illuminated. Such a process would necessarily preclude any discrimination in the responses of the innervated and denervated cells, for both types of melanophores should respond simultaneously to a blood-soluble substance which acts directly upon chromatophores. The matter was put to test by injecting into a totally pale specimen 0.2 cc. of commercial pituitrin:

PROTOCOL II (AUGUST 13, 1934)

Injections were made at 11:25 P.M., and at 11:50 P.M. the fishes were decidedly darker. The bands darkened at the same pace as the rest of the animal. At 12:30 A.M. the fishes were uniformly very dark.

Both innervated and denervated melanophores expanded simultaneously and completely. Since illumination of blinded animals evokes a rapid expansion of normal melanophores, and leaves the denervated cells completely contracted for some time, it would seem that the pituitary could not be the agency responsible for the reactivity of the melanophores of blinded animals. This conclusion was confirmed by testing the reactions of blinded animals whose pituitary glands had been removed. As a matter of fact, von Frisch in his admirable work in melanophore physiology had performed this experiment twenty-five years ago, and his results were fully substantiated. Several blinded animals were maintained in total darkness and their reaction tested a half-dozen times to insure that they were typical specimens. They were then hypophysectomized and returned to the darkroom. On the next day the tests were repeated. When illuminated they were found to be as pale as control specimens kept alongside in another dish, and within several minutes they turned quite dark. Again, this dark coloration was not as extreme as that which occurs when blinded (not hypophysectomized) animals are illuminated. Repeated tests showed that the blinded hypophysectomized fishes darkened to illumination, and paled after cessation of illumination.

We may now consider again the peculiar situation described previously, namely, the situation found in blinded animals kept in darkness where the innervated melanophores were contracted and the denervated melanophores were expanded. This same condition was found in similarly prepared but hypophysectomized specimens. Such specimens were uniformly pale except for the denervated band which appeared distinctly black, although not as black as that of blinded normal animals.[3] We are therefore left with no better explanation for this puzzling situation than that which has already been advanced, namely, the condition of a totally unstimulated denervated melanophore is one of pigment dispersion.

The receptor involved in the sensitivity of blinded catfishes to light was not investigated. Both von Frisch (1911) and Scharrer (1928) declared that the light receptor was situated in the vicinity of the diencephalon, but were unable to locate it precisely. Franz (1912) suggested that the so-called "neuropendymzellen im Thalamusependym" were light receptors in goldfishes. Scharrer (1932), impressed with the cytological secretory appearance of this region, wrote "im Reaktionsablauf sind wahrscheinlich nervöse und innersekretorische Vorgänge verknüpft." The peripheral route, however, does not seem to be hormonal, as the above experiments have indicated.

4. *Summary.*—All but one of the observations just described are easily understood. The nervous system directed by optic impulses is the controlling element in the chromatic responses of the catfish. This control Parker designates as neurohumoral since the eyes may eventually relegate the impulses they receive from the background throughout areas of denervated pigment cells. Blinded catfishes run the full gamut of pigmentary response from pigment concentration to pigment dispersion, and while this is a result of nervous stimulation, it does not include denervated cells. Neurohumoral responses are therefore normally lim-

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[3] The reason that innervated and denervated regions of integument in hypophysectomized animals are not as black to the naked eye as those of normal animals was not determined. Although the condition of the bands and the normal areas of melanophores were described as dark or pale, microscopic examinations were made in most cases with the purpose of determining the relative degree of expansion or contraction of the dermal melanophores. Such factors as the behavior of the xanthophores and the epidermal melanophores which contribute to the external coloration of the animal await further investigation.

ited to animals with eyes. The pituitary is not an indispensable agency for melanin dispersion, yet it aids melanophore expansion especially for denervated cells, and by its presence hinders melanophore contraction resulting from efferent nerve impulses. Melanophores when totally unstimulated (denervated cells in blinded animals kept in darkness) remain indefinitely expanded. This condition seems to be due to an intrinsic property of a denervated cell. The sole observation which is not explicable by any of these mechanisms is the quick pallor of denervated bands and normal areas of the integument in emotionally disturbed animals as noted in Protocol I and elsewhere in the previous account. This matter may now be considered in detail.

C. *Excitement Pallor*

The notion that concentrating hormones similar to adrenalin (or probably adrenalin itself) may play a part in the color changes of fish is by no means novel. In 1911 von Frisch noticed that a dark denervated area in *Phoxinus* blanched quickly following excitation of the animal. Scharrer (1928) and Giersberg (1930) inclined to the view that such pallor must be due to endocrine factors acting directly on the chromatophores or on their nerve endings. Meyer (1931) was able to induce paling of dark specimens of *Gobius* and *Pleuronectes* by injecting serum from light specimens, and in the same year Smith (1931) established the necessity of the blood supply for the paling of dark ophthalmic areas (denervated) in *Phoxinus*. He concluded furthermore that the substance which effected a pallor in denervated regions is a hormone suspected to be adrenalin.

Observations of a somewhat similar nature were made by Bray (1918) in the catfish *Ameiurus*. Bray " raised sensitive individuals to a high pitch of nervous excitation," and found that the melanophores contracted to the maximum extent and remained in this state for a protracted period. This behavior Bray attributed to the eyes, for following etherization and removal of the eyes the melanophores became expanded, even if the fish was kept in the light. From such experimentation the conclusion was advanced that " there is a suggestion of the secretion of a hormone under certain conditions and of its influence on the melanophores."

I have frequently observed that catfishes paled when they were obviously disturbed by movements made in capturing them. Dark caudal bands which these animals possessed also bleached and remained in this condition several hours after the fish as a whole had darkened. Eventually, the band regained its former tint. The temporal sequence of this process was as follows: Within two to five minutes after excitation of the animal, paling occurred throughout the fish except in the denervated band which retained its dark tint for from five to ten minutes longer. The band then faded rapidly and completely. Meanwhile, the body of the fish began to resume its initial dark color, but the band remained in a state of protracted pallor for from three to five hours. At the end of this time, the denervated caudal melanophores began to expand slowly (the animal being kept in black surroundings) and were fully expanded some three hours later.

Similar effects were evoked by electrical stimulation of the animal through the roof of the mouth, medulla or anterior end of the spinal cord. Electrical stimulation for two minutes resulted in a concentration of melanophore pigment in normal regions of the integument, and some five minutes later the denervated melanophores, dermal and epidermal, contracted in the manner already described (Figs. 10, 11, 12). Stimulation was carried out by the unipolar method, a lead plate on which the animals were placed being employed as a lead-off. Direct current (two volts, five amperes) was transformed by a Harvard inductorium. Animals placed in water between two stimulating electrodes also showed the same responses. These reactions were furthermore produced either by mechanical or electrical excitation of blinded specimens. The caudal band paled and darkened in the same period of time characteristic for normal animals, and thus contrary to Bray's contention that the eyes are necessary for pallor following excitation, both normal and blinded animals show the same reactions.

This rapid pallor of denervated sectors is apparently different from neurohumoral paling described by Parker. Since in all of the preparations the denervated areas were vascularized as well as the normal areas of the tail, and since the blood stream is the only quick avenue of approach to denervated cells, it was natural to suspect the blood stream as the agency responsible for the pallor following excitation. The temporal relations of the behavior of the innervated and denervated cells furthermore bring to mind the adrenalin vasodilator mechanism in mammals. A series of experiments was therefore directed toward a demonstration of the similarity between these two sets of observations. The experiments were divided into three groups: (1) a study of the reactions to adrenalin of innervated and denervated cells in fishes adapted to various backgrounds, (2) a comparison of the results with adrenalin with those produced by electrical stimulation, (3) a study of the blood of electrically stimulated animals.

1. *The Effect of Adrenalin.*—In the extensive number of papers which have dwelt upon the action of adrenalin on chromatophores, no

attention has been given to the "after-effects" of this substance on either the innervated or denervated cells. Thirty regenerating specimens were used in this series. The dosage of adrenalin chloride (Parke, Davis and Company) injected was 0.2 cc. of a solution diluted one part adrenalin to ten thousand parts water. The results are given in Table II.

TABLE II

Response of melanophores to intraperitoneal injections of adrenalin.

Condition of areas when injection was made		Time in minutes of response by concentration of pigment	Background to which fishes were returned	Time in hours of appearance of darkening "after-effects"	
Innervated	Denervated	Innervated and denervated		Innervated	Denervated
Black	Black	(10–21)	Black	3 hours	6 hours
Black	Black	(10–21)	Intermediate	3 hours	7 hours
Black	Black	(6–30)	White	Faint darkening[*]	No darkening
Light	Light	No perceptible change	White	No darkening	No darkening
Light	Light	No perceptible change	Black	4 hours	8½ hours

[*] This slight darkening was observed occasionally 2 hours after injection was made. It was only of short duration, however, for the animal soon paled in accordance with the white environment.

2. *The Effect of Electrical Stimulation.*—Fifteen regenerating specimens constituted the experimental material for this series. Results are arranged in tabular form in Table III.

TABLE III

Responses of melanophores to electrical stimulation of the roof of the mouth.

Condition of areas when stimulation was applied		Time of response by concentration of pigment		Background to which fishes were returned	Time of appearance of darkening "after-effects"	
Innervated	Denervated	Innervated	Denervated		Innervated	Denervated
Black	Black	1–2 minutes	4–11 minutes	Black	6 minutes	2–5 hours
Black	Black	1–2 minutes	4–11 minutes	Intermediate	5 minutes	2–5 hours
Light	Light	No change	No change	White	Darkening in 7 minutes[*]	No darkening
Light	Light	No change	No change	Black	Darkening in 8 minutes	4 hours
Black	Black	1–2 minutes	10 minutes	White	5 minutes. Slight darkening[*]	No darkening

[*] These darkenings were observed occasionally. They were only of short duration and the animals soon paled in accordance with the white background.

The more important results listed in the tables are as follows:

(1) Adrenalin—Denervated and innervated melanophores contract simultaneously following administration of adrenalin. Denervated cells once contracted by adrenalin never expand unless, following injection, the animals are returned to a black background. Innervated regions of integument sometimes darken slightly, despite the fact that the animal is maintained in white surroundings. There is thus a differential after-effect of adrenalin upon innervated and denervated melanophores.

(2) Electrical Stimulation—Denervated cells contract ten minutes after the innervated cells have responded. Such contracted denervated melanophores never expand unless, following stimulation, the animal is placed on a black background. The innervated melanophores always expand slightly following removal of stimulation, despite the fact that the animal is maintained in white surroundings. Thus after electrical stimulation there is a similar differential after-response of innervated and denervated melanophores. (Compare Fig. 9 with Fig. 12.)

The reactions of the innervated and denervated melanophores of catfishes to electrical stimulation, to adrenalin injections in proper doses, and to excitation produced mechanically are practically identical. Furthermore, they are not different from the responses of innervated and denervated blood vessels and other mammalian tissues (intestinal loops) subjected to treatment with adrenalin (Hartman and McPhedran, 1917*a*, *b*; Meltzer and Meltzer, 1903*a*, *b*, *c*). One is tempted to explain these results by assuming that excessive stimulation, electrical or otherwise, brings about a rapid nervous contraction of the innervated cells and a somewhat slower humoral contraction of the denervated cells. The humoral effect appears to be due to a substance which shares some physiological properties of adrenalin. Parallelism of tissue response, however, is no absolute criterion for the identification of an unknown material. The failure of known responses to occur in animals from which a suspected organ (the adrenals, in this case) is removed would be an important point in the elucidation of these psychic responses. Unfortunately, adrenalectomy in the catfish does not seem possible, and evidence must be obtained by indirect methods. The blood of excited animals was therefore investigated.

3. *Experiments with the Blood of Electrically Stimulated Fishes.*—Injections of 0.5 cc. of blood serum, or defibrinated blood, obtained from electrically stimulated fishes, were made under the skin near the dorsal fins of normal black-adapted animals. In twenty-two trials only three animals showed light areas at the point of injection. Although these injection experiments met with little success, the reverse procedure of excluding the blood supply to the tail proved more illuminating. Smith

(1931) had already shown that stimulation of *Phoxinus,* whose hearts had been previously removed, did not produce pallor of the denervated ophthalmic area. Similar results were obtained with the denervated caudal band in the catfish. While the catfish, whose heart had been previously removed, paled during electrical stimulation, the denervated area did not blanch in the time it would have blanched in a normal animal. The tail of a regenerating specimen was cut off distal to the posterior dorsal fin, thus removing the blood supply and all internal organs anterior to the region of the posterior dorsal fin. Stimulation at the base of the spinal column brought about a concentration of the innervated caudal cells in the usual time, but had no effect upon the expanded denervated cells. The rapid blanching of the denervating band in emotionally disturbed, or electrically stimulated animals seems to be due to a substance carried through the blood stream, and is therefore distinct from that which Parker has designated " neurohumoral contraction."

4. *The Effect of Electrical Stimulation in Blinded and Hypophysectomized Animals.*—Several tests, executed with no great detail, were performed on blinded regenerating specimens. Injections of adrenalin or electrical stimulation of blinded regenerating specimens produced responses entirely similar to those already described for normal fishes. The denervated bands always darkened from four to eight hours after the innervated melanophores had expanded following injection or stimulation.

Przibram (1932) published an account of experiments which attempt to harmonize conflicting effects of pituitary preparations on fish melanophores. Spaeth (1918), Matthews (1933), Hewer (1926*b*), Odiorne (1933), and Wyman (1924) reported melanophore contraction following pituitary treatment; Abolin (1925), Giersberg (1930), and Parker (1934*a*) found that pituitary injections induced melanophore expansion. Przibram brought these conflicting results together by showing that weak dosages of pituitary hormones induced melanophore expansion, but that stronger dosages evoked contraction. These findings are entertaining, and the possibility that the rapid pallor of the denervated bands may be due to an enormous secretory outburst of the pituitary gland following stimulation, electrical or mechanical, is plausible. This possibility is voided by the results obtained from the stimulation of isolated tails, and even more ruled out by the fact that the electrical stimulation or mechanical excitation of hypophysectomized animals brought about the same responses found in normal animals.

IV. Discussion

The experiments of Smith (1931), Parker (1934*a*), and those reported in the preceding pages show that several mechanisms may operate in the induction of chromatic responses in a particular animal. In the catfish the nervous system is the dominant mechanism, but humoral materials borne in the blood stream support and supplement it. The humoral and nervous action on melanophores differ in that hormonal action is slower and in that both innervated and denervated cells are primarily affected simultaneously. The humoral material which under special states induces pigment concentration has been shown to exhibit some properties characteristic of adrenalin. Such states have been called emotional, and the pallor resulting from this type of excitation has been termed excitement pallor (Redfield, 1918). Excitement pallor, however, has not only been completely denied by Zoond and Eyre (1934) for reptiles, but the senior author (Sand, 1935) has taken the diametrically opposite stand, namely, that excitement in reptiles results not in pallor but in darkening.

This aspect of melanophore physiology is therefore in a rather confused state, and the problem can scarcely be considered closed. The evidence that the adrenal glands are effective in the production of excitement pallor in reptiles comes chiefly from Redfield's work, which was accepted for some time by Hogben, but later denied by Hogben and Mirvish (1928*b*), Zoond and Eyre (1934), and Sand (1935). These investigators studied the African chameleon and were unable to confirm Redfield's findings in the horned toad. The toad and the Cape chameleon differ apparently in this respect. That two reptiles are not identical in their chromatic behavior is not really surprising, for when one considers the chromatic responses of the fishes one encounters an even greater variability. It is therefore difficult to maintain that the comparative chromatic apparatus is the same for members of a single class, and even more so for two classes of animals. Impressed with the universality of the anatomical basis and the bionomic aspect of color changes, Sand attempts a comprehensive equation for chromatic activity in fishes and reptiles and in so doing eliminates the possibility that the adrenal glands may be concerned with pigmentary activity.[4] The equation is not complete for all reptilian forms for Sand fails to include Noble and Bradley's (1933) finding that hypophysectomy results in a complete pallor of *Hemidactylus*. Furthermore, the existence of endocrine mechanisms for melanophore activity in some teleosts offers as much of an obstacle for a universal equation of color changes in fishes as Redfield's data do for an all-nervous control in reptiles. The rapid blanching of denervated areas of skin in *Phoxinus* and *Ameiurus* seems to be explainable best on the assumption that a contracting substance reaches the experimental area through the blood stream. The assumption is strengthened by the fact that the denervated area does not blanch when the blood supply is cut off. Moreover, there are certain indications that the contracting substance is similar to adrenalin in pharmacodynamic effect. This is as far as the evidence goes for there is no proof that it is actually adrenalin, nor that, with the exception of Redfield's experiments with adrenalectomized toads, it is produced by the chromaffin tissue. These points must await further investigation, and the question of an adrenal influence in the color changes of fishes and reptiles is still open.

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[4] Sand bases his views on Zoond and Eyre's disclosure of several inconsistencies in Redfield's data. It seems, however, that Zoond and Eyre have dealt with the exceptional cases in Redfield's data rather than with the more crucial experiments.

While the nature of the contracting blood-soluble material remains obscure, the phenomena with which this substance has been associated are of wider occurrence in fishes than in reptiles. Pouchet (1876) noted that excitement and fright resulted in the formation of dark spots on a pale turbot, an observation which Schaefer (1921) and Sumner (1911) also record for flatfishes. The stingfish darkened (von Frisch, 1912) but *Trigla,* the gurnard, and *Phoxinus,* the minnow, turned pale. My observations on *Holocentrus,* the squirrel fish of Bermuda, indicate that excitement results in the pallor of an otherwise red fish. These are several of the cases which may be cited, but it is apparent that there is no uniformity of psychic response. In those animals in which a general pallor occurred it must be remembered that no detailed discrimination, if any, was made between the reactions of the innervated and the denervated cells. Consequently, a comparison with the catfish cannot be made, for such general pallor following excitation of a fish may be due only to a nervous contraction of its melanophores. A denervated region of integument is necessary in order to determine whether excitation pallor occurs in denervated regions. If the denervated area shared the pallor following excitation, the agency must therefore be non-nervous and would be comparable to that which Redfield has advanced for the horned toad.

The occurrence of dark spots in frightened fishes, and a more general darkening of others are even further removed from the possibility of unifying psychic chromatic responses, yet one of my observations in the catfish may be of some interest. I have excited catfishes which possessed black caudal bands but which were otherwise totally pale. At the end of ten minutes of mechanical excitation, the caudal bands were completely blanched but the innervated regions of skin were darker than the band and much darker than they had been before excitation. If the mechanism advanced for psychic response in the catfish is plausible, the darkening of normal areas of the integument may be a secondary effect brought about by the modification of the action of an adrenalin-like substance by the nervous system, and in the case of flatfishes, this secondary effect may be peculiarly restricted to small areas of the integument.

V. Conclusions

The factors which bring about changes in distribution of melanophore pigment are many. The eyes, as has been known for a long time, are the controlling elements for the adaptation of animals to black or white backgrounds. The control of the eyes is through the nervous system; yet Parker's work has shown that denervated cells may also respond to backgrounds, but this response occurs more slowly. The eyes, while essential for background adaptation, are not indispensable for the process of pigment migration in blinded but otherwise normal animals. Blinded animals may respond by full pigment concentration or dispersion, and while this response is nervous, it does not eventually include regions of denervated melanophores. Denervated melanophores of blinded fishes kept in darkness are expanded, despite the concentrated state of the innervated melanophores. Pale denervated bands become darker in blinded animals exposed to light, but there is no reason to suppose that this response is different from that which occurs when blinded specimens are kept in darkness. " Neurohumoral " responses therefore do not occur in blinded animals. The pituitary gland is not indispensable for expansion of innervated cells, but denervated cells are affected by pituitary insufficiency in that they do not readily attain a condition of full expansion in black-adapted hypophysectomized animals. Denervated melanophore areas bleach rapidly following excessive stimulation, electrical or mechanical. The agency responsible for such pallor may be a substance similar to adrenalin in pharmacodynamic effect.

LITERATURE CITED

Abolin, L., 1925. Beeinflussing des Fischfarbwechsels durch Chemikalien. I. Infundin- und Adrenalinwirkung auf die Melano- und Xanthophoren der Elritze (*Phoxinus lævis* Ag.). *Arch. mikr. Anat.,* **104**: 667.

Abramowitz, A. A., 1935. Regeneration of chromatophore nerves. *Proc. Nat. Acad. Sci., Washington,* **21**: 137.

Bray, A. W. L., 1918. The reactions of the melanophores of Amiurus to light and to adrenalin. *Proc. Nat. Acad. Sci., Washington,* **4**: 58.

Franz, V., 1912. Beitrag zur Kenntnis des Ependyms im Fischgehirn. *Biol. Centralbl.,* **32**: 375.

von Frisch, K., 1910. Ueber die Beziehungen der Pigmentzellen in der Fischhaut zum sympathischen Nervensystem. *Festschr. R. Hertwig.,* **3**: 15.

von Frisch, K., 1911. Beiträge zur Physiologie der Pigmentzellen in der Fischhaut. *Pflüger's Arch. ges. Physiol.,* **138**: 319.

von Frisch, K., 1912. Über farbige Anpassung bei Fischen. *Zool. Jahrb., Abt. allg. Zool. Physiol.,* **32**: 171.

Giersberg, H., 1930. Der Farbwechsel der Tiere. *Forsch. und fortschr.,* **6**: 450.

Giersberg, H., 1931. Der Farbwechsel der Fische. *Zeitschr. vergl. Physiol.,* **13**: 258.

Giersberg, H., 1932. Der Einfluss der Hypophyse auf die farbigen Chromatophoren der Elritze. *Zeitschr. vergl. Physiol.,* **18**: 369.

Hartman, F. A., and L. McPhedran, 1917*a*. Further observations on the differential action of adrenalin. *Am. Jour. Physiol.,* **43**: 311.

Hartman, F. A., and L. McPhedran Fraser, 1917*b*. The mechanism for vasodilatation from adrenalin. *Am. Jour. Physiol.,* **44**: 353.

Hewer, H. R., 1926*a*. Studies in colour changes of fish. II. An analysis of the colour patterns of the dab. III. The action of nicotin and adrenalin in the dab. IV. The action of caffeine in the dab, and a theory of the control of colour changes in fish. *Phil. Trans. Roy. Soc., London, B,* **215**: 177.

Hewer, H. R., 1926*b*. Studies in colour changes of fish. I. The action of certain endocrine secretions in the minnow. *Brit. Jour. Exper. Biol.,* **3**: 123.

Hogben, L., and L. Mirvish, 1928*a*. Some observations on the production of excitement pallor in reptiles. *Trans. Roy. Soc. So. Africa,* **16**: 45.

Hogben, L., and L. Mirvish, 1928*b*. The pigmentary effector system. V. The nervous control of excitement pallor in reptiles. *Brit. Jour. Exper. Biol.,* **5**: 295.

Matthews, S. A., 1933. Color changes in Fundulus after hypophysectomy. *Biol. Bull.,* **64**: 315.

Meltzer, S. J., and C. Meltzer, 1903*a*. A study of the vasomotor nerves of the rabbit's ear contained in the third cervical and in the cervical sympathetic nerves. *Am. Jour. Physiol.,* **9**: 57.

Meltzer, S. J., and C. Meltzer, 1903*b*. The share of the central vasomotor innervation in the vasoconstriction caused by intravenous injection of suprarenal extract. *Am. Jour. Physiol.,* **9**: 147.

Meltzer, S. J., and C. Meltzer, 1903*c*. On the effects of subcutaneous injection of the extract of the suprarenal capsule upon the blood-vessels of the rabbit's ear. *Am. Jour. Physiol.,* **9**: 252.

Meyer, E., 1930. Ueber die Mitwirkung von Hormonen beim Farbwechsel der Fische. *Forsch. und fortschr.,* **6**: 379.

Meyer, E., 1931. Versuche über den Farbwechsel von Gobius und Pleuronectes. *Zool. Jahrb., Abt. allg. Zool. Physiol.,* **49**: 231.

Noble, G. K., and H. T. Bradley, 1933. The relation of the thyroid and the hypophysis to the molting process in the lizard, Hemidactylus brookii. *Biol. Bull.,* **64**: 289.

Odiorne, J. M., 1933. The effects of the pituitary hormones on the melanophores of fishes. *Proc. Nat. Acad. Sci., Washington,* **19**: 745.

Parker, G. H., 1932. Humoral agents in nervous activity, with special reference to chromatophores. Cambridge.

Parker, G. H., 1934*a*. Color changes in the catfish Ameiurus in relation to neurohumors. *Jour. Exper. Zoöl.,* **69**: 199.

Parker, G. H., 1934*b*. The prolonged activity of momentarily stimulated nerves. *Proc. Nat. Acad. Sci., Washington,* **20**: 306.

Parker, G. H., 1934*c*. Neurohumors as activating agents for fish melanophores. *Proc. Am. Phil. Soc.,* **74**: 177.

Parker, G. H., F. A. Brown, Jr., and J. M. Odiorne, 1935. The relation of the eyes to chromatophoral activities. *Proc. Am. Acad.,* **69**: 439.

Pouchet, G., 1876. Des changements de coloration sous l'influence des nerfs. *Jour. Anat. et Physiol. norm. et path.,* **12**: 1–90, 113–165.

Przibram, H., 1932. Einfluss von Infundin- und Adrenalindosierung auf die Färbung unserer Frösche, Rana esculenta L., R. fusca Rösel, Hyla arborea L. *Zeitschr. vergl. Physiol.,* **17**: 565.

Redfield, A. C., 1918. The physiology of the melanophores of the horned toad Phrynosoma. *Jour. Exper. Zoöl.,* **26**: 275.

Sand, A., 1935. The comparative physiology of colour response in reptiles and fishes. *Biol. Rev.,* **10**: 361.

Schaefer, J. G., 1921. Beiträge zur Physiologie des Farbenwechsels der Fische. I. Untersuchungen an Pleuronectiden. *Pflüger's Arch. ges. Physiol.,* **188**: 25.

Scharrer, E., 1928. Die Lichtemfindlichkeit blinder Elritzen. (Untersuchungen über das Zwischenhirn der Fische I.) *Zeitschr. vergl. Physiol.,* **7**: 1.

Scharrer, E., 1932. Die Sekretproduktion im Zwischenhirn einiger Fische. III. *Zeitschr. vergl. Physiol.,* **17**: 491.

Smith, D. C., 1931. The influence of humoral factors upon the melanophores of fishes, especially Phoxinus. *Zeitschr. vergl. Physiol.,* **15**: 613.

Smith, D. C., and M. T. Smith, 1934. Observations on the erythrophores of Scorpæna ustulata. *Biol. Bull.,* **67**: 45.

Spaeth, R. A., 1913. The physiology of the chromatophores of fishes. *Jour. Exper. Zoöl.,* **15**: 527.

Spaeth, R. A., 1918. Concerning a new method for the standardization of pituitary extract and other drugs. *Jour. Pharm. Exp. Ther.,* **111**: 209.

Sumner, F. B., 1911. The adjustment of flatfishes to various backgrounds; a study of adaptive color change. *Jour. Exper. Zoöl.,* **10**: 409.

Wyman, L. C., 1924. Blood and nerve as controlling agents in the movements of melanophores. *Jour. Exper. Zoöl.,* **39**: 73.

Zondek, B., 1935. Chromatophorotropic principle of the pars intermedia of the pituitary. *Jour. Am. Med. Assoc.,* **104**: 637.

Zoond, A., and J. Eyre, 1934. Studies in reptilian colour response. I. The bionomics and physiology of the pigmentary activity of the chameleon. *Phil. Trans. Roy. Soc., London, B,* **223**: 27.

CUTANEOUS MELANOSIS IN LUNGFISHES (LEPIDOSIRENIDÆ)

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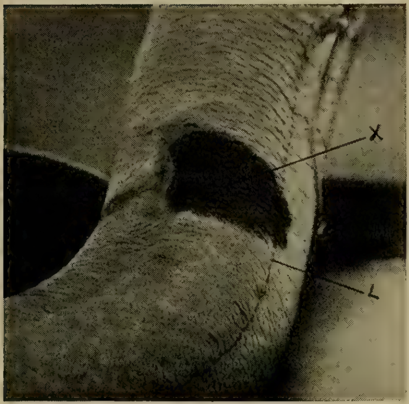
During the past two years an opportunity has been afforded to observe two instances of a localized cutaneous melanosis occurring in lungfishes of the New York Aquarium. Both fishes were of the species *Lepidosiren paradoxa Fitzinger,* and had been caught in the Amazon River. On arrival in the New York Aquarium, the skin in each instance presented a normal dark slate color without any pigmented patches. The onset of pigmentation was regarded therefore as a spontaneous one.

Lungfish *A* (Plate I, Fig. 1) developed a slightly raised intensely black patch about an inch square in the right dorsal region above and encroaching upon the lateral line. It was situated approximately three inches from the head. Three months later, a piece of tissue a half-inch in length was removed for histological study, and at the same time a piece of the normal skin was excised for purposes of control. The wound in the normal skin regenerated without any unusual pigmentation, but the wound made in the melanotic region healed as skin with intensely black coloring. The fish remained alive for over a year following the biopsy without any further changes in the skin, the melanotic patch remaining the same size as originally seen.

Lungfish *B* showed a similar but slightly smaller black pigmented area in the right dorsal region above the lateral line about five inches from the head. This entire pigmented area was excised with a wide margin. Regeneration of the wound occurred in the course of the next four weeks, the regenerated skin appearing as normal looking skin without intensification of pigment.

Under aquarium conditions the skin of *Lepidosiren paradoxa* shows normally an epidermis composed of from four to six layers of epithelial cells, somewhat cuboidal near the free surface and polygonal or round near the basement membrane (Plate I, Fig. 3). Large mucous cells lie scattered throughout the epithelium, containing droplets of mucus which distend the cell and displace the nucleus toward the base. The epidermis rests upon a well developed basement membrane, below which lies a



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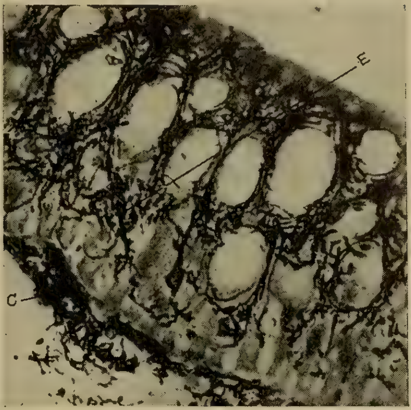

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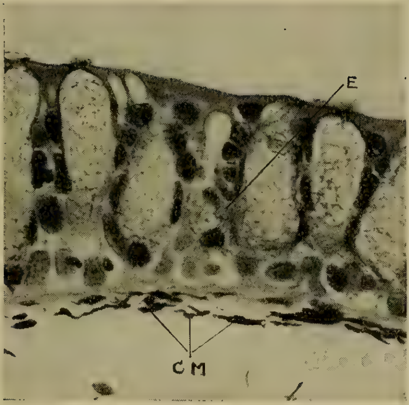

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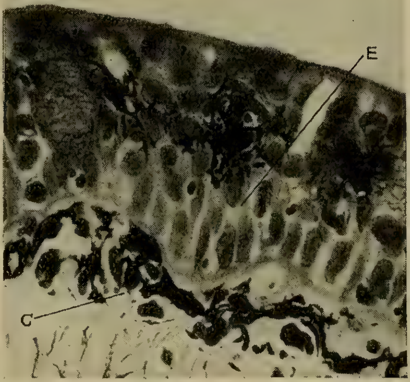

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Plate I

EXPLANATION OF PLATE I

Fig. 1. *Lepidosiren paradoxa* (lungfish *A*) with pigmented patch (*X*) in the right dorsal region encroaching on the lateral line (*L*).

Fig. 2. Photomicrograph of melanotic tissue removed from lungfish *A*, showing a hyperplasia of melanophores infiltrating thickened epidermis (*E*). Numerous melanophores in the corium (*C*). Rogers' silver stain, counterstained faintly with erythrosin. × 250.

Fig. 3. Normal skin removed from lungfish *B*. *E*. epidermis; *CM*. corial melanophores. Hematoxylin and eosin. × 250.

Fig. 4. Tissue removed from melanotic patch of lungfish *B*. Mild increase of melanophores in corium *C* and a moderate infiltration of epidermis *E* by melanophores. Hematoxylin and eosin. × 250.

loosely arranged corium composed of interlacing fibrous tissue containing nerves and capillaries. Under normal conditions of pigmentation, the corium presents a moderate number of melanophores lying close under the basement membrane. Only occasionally are melanophores found among the epithelial cells of the epidermis.

The histological examination of the melanotic patches of skin removed from both the lungfishes, however, showed a greatly increased number of melanophores not alone in the corium but also infiltrating a somewhat thickened epidermis. These pathologic changes were noted particularly in lungfish *A* (Plate I, Fig. 2). A dense interlacing network of melanophores lies throughout all the layers of the epidermis. Intertwining dendritic processes of melanophores penetrate between epithelial cells and surround mucous cells, reaching actually to the free surface of the epithelium. The corium shows an increase over the normal numbers of melanophores, but adjacent muscle tissue was not invaded by pigment cells. The hyperplasia of melanophores was not associated with any leucocytic reaction. We found no evidence of parasites in the sections of diseased tissue examined. The melanophore infiltration of the epidermis of lungfish *B* was less extensive in character (Plate I, Fig. 4).

Discussion

Abnormal black pigmentation of the skin of fishes seems to depend on both genetic and post-embryonal factors. It may result from a more or less prolonged or permanent expansion of melanophores, or from an actual increase in the melanophores of the skin. Researches of Ballowitz (1893), von Frisch (1911) and other investigators have indicated that pathways exist between the brain through pigment motor nerve fibers to the sympathetic system, and from here by means of the peripheral nerves of the skin to the melanophores, affecting their contraction and expansion. Interruptions of these pathways by pathologic processes may result in an unusual black pigmentation of the skin produced by expansion of melanophores. Pathologic pigmentation of the skin caused by an increase in the number of melanophores occurs, for example, when certain parasitic larvæ gain access to the skin and become encysted. Mechanical trauma and X-raying have produced eruptions of corial melanophores and cutaneous pigmentation in the goldfish under experimental conditions (Smith, 1931, 1932). Yet in neither of the lungfishes exhibiting melanosis, kept for a long period of time in individual tanks, had there been any wounding of the skin.

Experimental studies covering a wide biological field attribute pigmentation to disturbances involving the endocrine system or enzyme activity. Further investigations of melanosis of lungfishes may yield more definite information in these directions. It is unlikely that this, a distinct hyperplasia of pigment cells as well as of the epithelium, could be the result of humoral activity. In the present observation on two lungfishes, the stimulus to pigmentation remains undetected.

LITERATURE CITED

BALLOWITZ, E., 1893. Die Nervenendigungen der Pigmentzellen, ein Beitrag zur Kenntnis des Zusammenhanges der Endverzweigungen der Nerven mit dem Protoplasma der Zellen. *Zeitschr. f. Wissenschaft. Zoöl.*, **56**: 673.

VON FRISCH, K., 1911. Beiträge zur Physiologie der Pigmentzellen in der Fischhaut. *Arch. f. ges. Physiol.*, **138**: 319.

SMITH, G. M., 1931. The occurrence of melanophores in certain experimental wounds of the goldfish (Carassius auratus). *Biol. Bull.*, **61**: 73.

SMITH, G. M., 1932. Eruptions of corial melanophores and general cutaneous melanosis in the goldfish (Carassius auratus) following exposure to X-ray. *Am. Jour. Cancer*, **16**: 863.

A STUDY OF THE ENVIRONMENTAL CONDITIONS IN A BOG POND WITH SPECIAL REFERENCE TO THE DIURNAL VERTICAL DISTRIBUTION OF GONYOSTOMUM SEMEN

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INTRODUCTION

This is a study of a round, bog pond ("Cedar Pond") situated near the Marine Biological Laboratory in Woods Hole, Massachusetts. Well protected from winds by a wooded, sphagnum bog, the pond is ideal for a study of the vertical distribution of organisms. Higher chlorophyll-bearing plants were almost absent from the pond, during the summer of 1935, but large numbers of a chlorophyll-bearing flagellate, *Gonyostomum semen,* were present. The vertical distribution of this flagellate, the morphology of which has been described by Drouet and Cohen (1935), was studied together with such environmental factors as O_2, CO_2, pH, temperature, and illumination at the surface of the water.

No extensive discussion of previous work on vertical migration is undertaken in this paper, first, since the group of organisms studied is one not ordinarily investigated, and second, since it has been established so thoroughly that no one interpretation is possible for the various groups of animals and plants or for individual members of the same species (Parker, 1902; Esterly, 1907, 1917, 1919; Utermöhl, 1924; Whipple, 1927; Kikuchi, 1930; Worthington, 1931; Southern and Gardiner, 1932; Clarke, 1934; and Welch, 1935), and, third, since comprehensive reviews of the subject may be found in Rose, Russell, Whipple, Kikuchi, Clarke, and Welch.

METHODS

The water samples, both for the study of organisms and for the chemical and physical tests, were collected with an Esmarch sampler which carries a 50-ml. sample bottle with a ground glass stopper. The temperature and pH readings of the water samples were made immediately after they were brought to the surface. Samples to be used for analysis for gas content were so collected that there were no air bubbles in the bottles, and they were kept packed in ice till the gas analyses were made in the laboratory. The gas analyses were finished within two or three hours.

The degree of illumination was measured in foot-candles at the surface of the water, using a Weston photometer which was so constructed as to measure illumination from a few foot-candles to several thousand.

Since no extreme accuracy for temperature readings was thought necessary, they were made with an ordinary laboratory thermometer.

A Youden hydrogen ion apparatus with quinhydrone electrodes was used for determining the pH values of the water, corrections being made for temperature.

The dissolved oxygen and the total carbon dioxide (Σ CO_2) values, corrected for the usual interfering conditions, and for the H_2S in some samples, were determined with a Van Slyke manometric apparatus. In the earlier part of the work determinations were made in duplicate, but after it was found that values were reproducible, duplicates were determined only in those cases where the results were questionable. The free CO_2 and bicarbonate CO_2 were calculated using the proper equation (Clark, 1928). The carbonate content was considered negligible.

The organisms were counted under a compound microscope using a Sedgwick Rafter counting cell and a Whipple disc ocular. As a rule, total counts were made and the results were recorded in round numbers.

Physical and Chemical Conditions in the Bog Pond[1]

A study of the temperatures of the water from the surface to the bottom on August 20 and 21 (Table I) shows clearly, as would be expected, that the surface water increases in temperature from 5 A.M. to 1 P.M. but that by 5 P.M. it has begun a decrease which continues till the next morning. These changes are evidently brought about mainly by changes in air temperature. A similar but much slighter effect could be detected down to the 2.0-meter depth.

The wide range in water temperature, namely, from over 30 to 11° C. from surface to bottom (Table I), made it necessary to correct the pH values of the water for temperature at various depths. Our studies of the water of this pond show that it is acid in reaction. From the surface down to a depth of 1.0 meter the pH value is low (Table I) and much like that of the sphagnum bog water which surrounds the pond, i.e., it is distinctly acid with a pH value of 4.0 to 5.0. It is evident from the similarity in acidity of the sphagnum bog water (Drouet and Cohen, 1935) to that of the upper meter stratum of the pond, and from the increasing pH value of the water of the pond passing downward below one meter, and from the existence of free communication between the sphagnum bog and the pond, that the upper meter of the pond water is largely of sphagnum bog origin. This conclusion supports the view of Jewell and Brown (1929).

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[1] The authors are pleased to acknowledge having received advice from Dr. Robert Stiehler of the Wilmer Institute of the Johns Hopkins Hospital concerning certain chemical problems in this investigation.

Below the 1.0-meter depth the pH values for the water increase to over 6.0 usually and sometimes to almost 7.0 (Table I). Such an increase toward the neutral point at the bottom shows clearly that the greater acidity in the upper one meter of the bog pond does not have its origin from the bottom.

A diurnal change in the pH values is known to occur in certain bodies of water, especially fresh water, due to a decrease in the amount of CO_2 which is used up in the photosynthetic activity of the green organisms, although some of the decrease may be due to an increase in the temperature of the water with the consequent liberation of CO_2 into the air. Such a result has been reported by Bergman (1921), Cowles and Schwitalla (1923), Philip (1927), and Welch (1935). A glance at Table I shows this in the deeper levels of the pond, but above 2.0 meters such a condition is not so striking, probably because the acid water of the sphagnum bog, part of whose acidity is known to be due to some conditions other than the presence of free CO_2, keeps the pH value low throughout the whole twenty-four hours (Cowles and Brambel, 1934).

The determination of the CO_2 in the water of the bog pond, using the Van Slyke apparatus, gives us the total CO_2 ($\Sigma\, CO_2$) content, i.e., the sum of the free CO_2, bicarbonate CO_2, and carbonate CO_2. However, the low pH values of the upper layers, which consist mainly of sphagnum bog water which has seeped into the pond, show that the total CO_2 values of these layers are almost entirely free CO_2, since especially the carbonate and also the bicarbonate tend to be greatly reduced at such pH values. Below the 0.5-meter level bicarbonate becomes more important and this is indicated, in general, by the higher pH values (Table I). Water at these levels is largely of subterranean origin. Correlated with this difference in origin, the 0.5-meter water is characterized by only a trace of carbonate and only a small amount of bicarbonate with a $\Sigma\, CO_2$ of 16.2 p.p.m. The water of the 1.0-meter level has still only a trace of carbonate but a greatly increased amount of bicarbonate with a $\Sigma\, CO_2$ of 80.5 p.p.m. This sudden and extensive difference in total CO_2 content of the water passing through what may be called the transition layer is the most striking characteristic of the pond.

A diurnal variation in the $\Sigma\, CO_2$ is indicated fairly well in the surface water and it may be noted that the $\Sigma\, CO_2$ is very largely free CO_2, owing to a low pH value resulting from some constituent other than CO_2 (Table I). The lowest Σ CO_2 value (almost entirely free CO_2) occurs in the late afternoon or early evening after the day's photosynthetic activity of *Gonyostomum* has about ended in the upper layers. On August 14 and 15 the same diurnal variation occurred. A similar condition at 0.5 meter existed on August 14 and 15 and very probably on August 20 and 21, but a very large irregularity in the 5 P.M. value on August 20 prevents us from drawing a positive conclusion concerning the latter observations.

The data show (Table I) that the free CO_2 content in general increases from the surface down to about 1.0 meter, as does also the total CO_2, and that the former constitutes a very large part of the latter. But beyond that depth the free CO_2 content decreases much although the total CO_2 content reaches its highest values at the bottom. Correlated with this relation, it is found that the bicarbonate CO_2 which is present in small amounts from the surface down to 1.0 meter, begins to increase in actual value, as well as to increase proportionally in value with the decrease in free CO_2. So in the upper layers the free CO_2 is far in excess of the bicarbonate CO_2 (Table I). At the bottom the bicarbonate CO_2 is far in excess of the free CO_2. But in passing toward the surface from the 1.5-meter layer to the 1.0-meter layer, where there is a distinct increase in acidity, which is indicated by the lower pH values, the shift from the condition in the lower layers to that in the upper ones occurs. Apparently in this region where the underlying real pond water, rich in bicarbonates, meets the more acid overlying water from the sphagnum bog, the acids react with the bicarbonates liberating CO_2 and the free CO_2 content of the water is thus increased and the bicarbonates decreased.

The dissolved free CO_2 of the surface water and in fact that of other levels is present in considerable amounts in the early morning (see also Welch, 1935), due to the respiration of organisms during the night, also to decomposition of organic matter in the water as well as at the bottom and likewise to the cessation largely, if not completely, of photosynthetic activity during the night. The decrease during the hours of the morning and usually during the early afternoon must be due to the photosynthetic activity of *Gonyostomum,* mainly, and to an increase in temperature which would tend to liberate some of the free CO_2 into the atmosphere. During the night photosynthesis ceases, or is greatly reduced and respiration increases, thus increasing the free CO_2 content of the water. The lowered temperature of the night helps to keep the CO_2 in solution (Table I).

The O_2 content of the water is about the same as that of small ponds

TABLE I

The diurnal and vertical distribution of Gonyostomum semen *in Cedar Bog Pond, Station D* (*see Fig. 2*) *on August 20 and 21, 1935, in relation to chemical and physical conditions.*

Time	Depth	Air temperature	Water temperature	pH	Total CO_2	Free CO_2	Bicarb. CO_2	Diss. O_2	Surface illumination	Gonyostomum
	meters	° *C.*	° *C.*		*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*foot-candles*	*per 2 ml.*
5 A.M.	0.0	23	24	4.7	5.8	5.3	0.5	5.8	350	100
	0.5		23	4.7	16.2	14.7	1.5	1.7		1400
	1.0		20	4.9	80.5	69.5	11.0	1.2		1000
	1.5		17	5.2	83.0	63.0	20.0	0.6		200
	2.0		14	5.2	66.5	50.5	16.0	1.3		1100
	3.0		11	5.8	101.7	44.8	56.9	0.3		600
9 A.M.	0.0	28	27	4.7	4.6	4.2	0.4	6.2	3100	0
	0.5		24	4.9	12.8	11.1	1.7	2.4		1600
	1.0		15	5.1	79.5	63.5	16.0	1.3		400
	1.5		15	5.9	78.0	30.2	47.8	1.3		400
	2.0		14	6.0	78.0	26.0	52.0	0.6		600
	3.0		11	6.1	89.0	25.3	63.7	1.3		300
1 P.M.	0.0	30	30	4.6	7.5	6.9	0.6	4.7	3200	0
	0.5		25	4.6	11.6	10.7	0.9	2.7		900
	1.0		20	4.7	64.5	58.6	5.9	6.1		800
	1.5		18	5.2	85.0	64.5	20.5	0.3		400
	2.0		15	6.2	101.0	24.3	76.7	1.0		500
	3.0		11	6.4	70.5	11.6	58.9	1.3		500
5 P.M.	0.0	26	30	4.0	3.5	3.4	0.1	7.3	1000	0
	0.5		23	4.6	58.6?	54.3?	4.3?	3.3		100
	1.0		22	4.6	52.0	48.2	3.8	1.2		1000
	1.5		18	5.0	85.0	70.8	14.2	0.6		1200
	2.0		15	6.3	75.0	15.0	60.0	1.2		600
	3.0		11	6.6	105.0	11.7	93.3	1.3		900
9 P.M.	0.0	22	28	4.5	4.0	3.8	0.2	8.2	0	0
	0.5		25	4.7	8.1	7.3	0.8	2.8		0
	1.0		21	4.8	62.5	55.5	7.0	0.3		500
	1.5		17	5.0	88.0	73.3	14.7	0.3		800
	2.0		13	6.0	76.0	25.4	50.6	1.3		900
	3.0		11	6.0	96.0	32.1	63.9	1.3		900
1 A.M.	0.0	23	26	4.0	4.6	4.5	0.1	6.8	0	0
	0.5		24	4.0	13.3	12.9	0.4	1.3		100
	1.0		21	4.5	71.5	67.3	4.2	1.7		600
	1.5		17	5.5	79.0	49.0	30.0	1.0		900
	2.0		14	5.6	77.0	43.0	34.0	0.7		1000
	3.0		11	6.1	96.0	27.3	68.7	0.7		500
5 A.M.	0.0	22	25	4.3	5.8	5.6	0.2	7.2	150	200
	0.5		24	4.3	7.5	7.2	0.3	4.1		800
	1.0		20	4.5	79.5	74.6	4.9	1.0		1100
	1.5		17	5.0	84.5	70.4	14.1	1.3		700
	2.0		13	5.0	75.0	62.5	12.5	1.0		700
	3.0		11	6.1	102.0	29.0	73.0	1.0		300

in general. It is highest at the surface and decreases in most cases rather uniformly with the depth. The low O_2 content in the deeper layers is probably due, as suggested by Welch (1935) for other waters, to the presence there of considerable amounts of organic matter, to other gases rising in the form of bubbles from the bottom and very probably to an oxygen-depleted subterranean water which enters the pond at the bottom. Ordinarily there is a sharp decrease from the surface to the 0.5-meter level after which the decrease is much less, more gradual, and somewhat irregular, as can be seen from Table I.

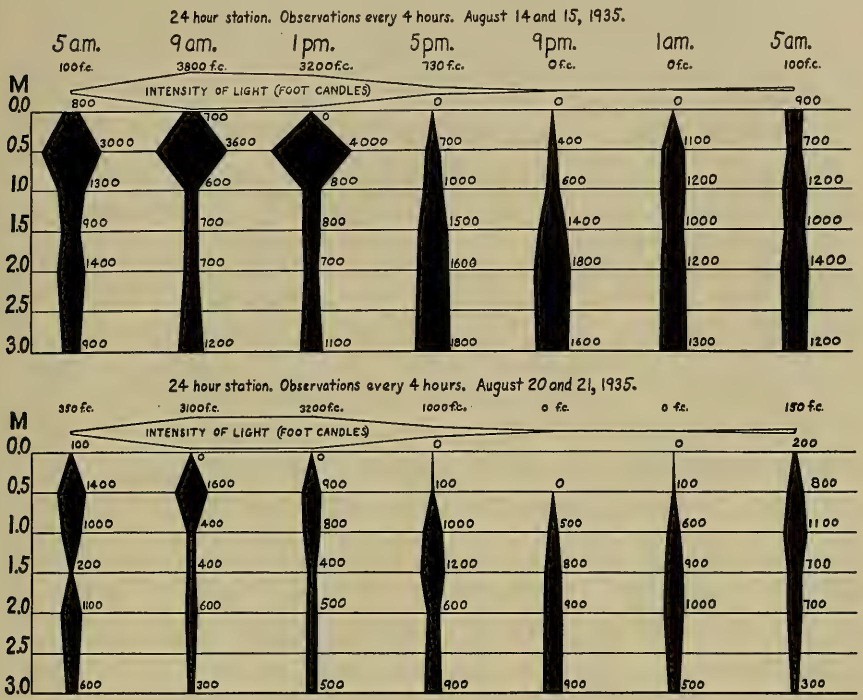

FIG. 1. This figure shows the vertical distribution of *Gonyostomum semen* from the surface to the bottom of Cedar Pond during two 24-hour periods. All the observations recorded in this figure are for the vertical distribution at Station *D*. In each observation the number of organisms in 2 ml. of water are shown. The degree of illumination in foot-candles at the surface of the water is shown for each observation.

A daily increase in dissolved oxygen has been found to occur on sunny days during the morning or morning and afternoon, especially at 0.5 meter (Table I). It is at the 0.5-meter depth that the maximum numbers of individuals of *Gonyostomum* (Fig. 1) were found during the morning hours and it is reasonable to suppose that the increase in O_2 is due largely to the photosynthetic activity of that organism, although another supposedly chlorophyll-bearing flagellate found associated with *Gonyostomum* may possibly be another small source. It will be noted from Table I that the oxygen content decreases fairly regularly at the 0.5-meter level a short time after the *Gonyostomum* individuals begin their descent to the lower layers during the late afternoon (Fig. 1).

The average of the dissolved oxygen values on August 14 and 15, for Station *D*, from surface to bottom, at 1 P.M., as compared with the average at 1 A.M., are 2.7 p.p.m. and 2.1 p.p.m. Similar averages for August 20 and 21 are 2.7 p.p.m. and 2.0 p.p.m. These differences between the O_2 values at 1 P.M. and 1 A.M. are presumably due in a large part to a change in the photosynthetic activity of *Gonyostomum*.

The dissolved O_2 of the water of this bog pond and especially of the surface and 0.5-meter levels is considerably depleted early in the morning, e.g., 1 A.M. (Table I), due to the respiration of organisms and oxidation of organic matter in the water and at the bottom. During the morning or the morning and afternoon the dissolved O_2 in the water increases as the result, mostly, of the photosynthetic activity of *Gonyostomum*. The rise of temperature during this time favors the escape of O_2, but notwithstanding this, the increase in O_2 or " oxygen pulse," as it is sometimes called (Welch, 1935), is distinctly evident.

The data obtained with a Weston photometer for the illumination at the surface of the water are shown in Fig. 1 and Table I. The relation of these values to the distribution of *Gonyostomum* will be taken up in the next section.

Distribution of Gonyostomum

On an exceptionally bright morning it was found that at seven different stations distributed along a line extending across the bog pond the largest counts of swimming individuals of *Gonyostomum* were at a depth of 1.0 meter (Fig. 2). Judging from this widespread similar vertical distribution in the brownish colored water of this pond, it would seem then that under the physical and chemical conditions of that day and that time of day, a 1.0-meter depth from the surface offered the optimum conditions for these organisms. On other somewhat less bright mornings the maximum numbers were found at a depth of 0.5 meter (Fig. 1), while on a cloudy morning the maximum counts were at the surface. Muttkowski (1918) finds that the level for optimum photosynthetic activity for green plants in Lake Mendota, which is a clear, uncolored lake, is between 3 and 5 meters. Juday and

Schomer (1935) obtained similar results, by different methods, for other lakes.

The dependence of *Gonyostomum* on light as a stimulation for migration upward is strongly indicated, as one can see from the results shown in Fig. 1. At 5 o'clock in the morning, with only 200 to 300 foot-candles of illumination at the surface of the water, the *Gonyostomum* individuals were distinctly massed in the region of 0.5 meter below the surface and at 9:00 in the morning and at 1:00 in the afternoon, with an increasing illumination resulting in 3,000 foot-candles, 0.5 meter still seemed to offer the optimum conditions. But even at 1:00 P.M., on August 20, the counts indicated downward migration, since the number of organisms at 0.5 meter has decreased a little, while at nearly all of the lower levels the numbers have increased. By 5 P.M., with a surface illumination of only a few foot-candles, the migration downward is indicated again by the higher counts at the lower levels and this condition continues at 9 P.M. when the photometer failed to show any appreciable illumination. It may be seen that in the case of both of the twenty-four-hour periods during which studies were made, at 5 P.M. and 9 P.M., the bottom (3.0 meter) counts were the largest when compared with those for any other time during the whole twenty-four hours at that depth. This increase in the lower layers with a corresponding decrease in the upper layers is almost positive proof that there has been an extensive migration or a quiescent sinking of the individuals of *Gonyostomum* toward the bottom. In this paper the bottom of the pond is spoken of as being at 3.0 meters—the lowest depth at which satisfactory

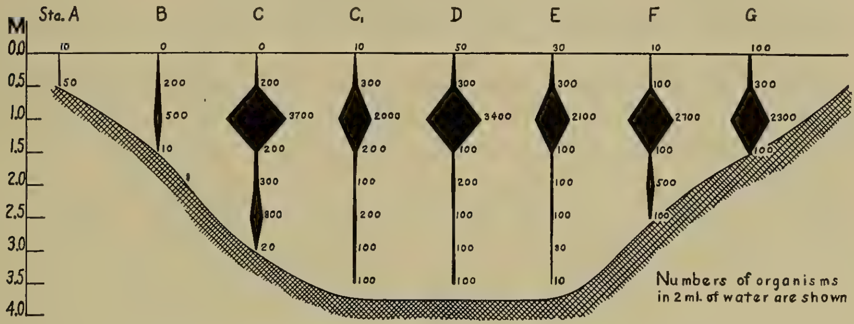
M
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Sta. A
10
50
B
0
200
500
10
C
0
200
3700
200
300
800
20
C₁
10
300
2000
200
100
200
100
100
D
50
300
3400
100
200
100
100
100
E
30
300
2100
100
100
100
30
10
F
10
100
2700
100
500
100
G
100
300
2300
100
Numbers of organisms
in 2 ml. of water are shown

FIG. 2. This figure deals with the vertical distribution of *Gonyostomum semen* from the surface to the bottom of Cedar Pond from 9 A.M. to 10 A.M. on August 25, 1935. The vertical distribution is shown at Stations *A*, *B*, *C*, *C*$_1$, *D*, *E*, *F*, and *G* which lie along a straight line extending across the middle of the pond. The day was exceptionally bright and there were no clouds.

samples could be taken in routine work. Between this depth and 3.5 meters, where there is a somewhat solid bed of decaying vegetation, there is a mush of dark brown mud and frequently of vegetation where, ordinarily, it is a very laborious process and one of questionable accuracy to make counts of organisms.

It is quite surprising to find that by 1 A.M. both on August 15 and August 21, individuals of *Gonyostomum* began to increase again in the upper layers and decrease at the lower levels (Fig. 1), indicating an upward migration. This migration, at 1 A.M., was more marked on August 15 than on August 21 and correlated with this difference, our records show that there was bright moonlight on the night of August 14–15, while on the night of August 20–21 there was much fog although at times the moon was to be seen. While our observations concerning the effect of moonlight on the behavior of *Gonyostomum* are meager, they suggest that these organisms react to the moonlight and migrate toward the surface, when there is moonlight during the early morning hours. Such a suggestion as to the cause of this behavior is supported by the observations just mentioned, concerning the illumination due to moonlight on two different nights, as compared with the numbers of *Gonyostomum* individuals to be found in the upper levels of this pond.

In the opinion of the authors, the observations made and our knowledge of the structure and behavior of *Gonyostomum* indicate that light is an important factor in the upward migration of that organism. It would seem that having spent several hours in darkness and, as a result, having probably become exceptionally sensitive to light, it tends to migrate upward till it reaches the proper combination of illumination, free CO_2 and O_2 content, and temperature, which are necessary for the required photosynthetic activity.

The downward movement in the early afternoon, sometimes as early as 1 P.M. when the light is still bright, is rather surprising. One might suspect that having carried on the process of photosynthesis long enough to satisfy their carbohydrate needs, the *Gonyostomum* individuals swam or sank quiescently toward the bottom. Drouet and Cohen's (1935) observation and our own have shown that they do sink to the bottom of jars in the laboratory.

We are dealing with a periodic phenomenon here—a migration to the upper levels during the morning and a movement toward the bottom in the afternoon and evening. The theory that this may be regulated by some internal condition of the organism, by some periodic physiological change which regulates the periodicity in behavior, must not be disregarded. There is considerable evidence to show, in the case of certain snails and green unicellular forms, that periodic changes in behavior which are correlated with definite periodic changes in the environment occur even when the organisms are no longer subjected to these changes in the environment. Mast (1920), studying a euglenoid form, *Lepocinclis texta,* found a periodic reaction toward light and it is very probable that the periodic movement of *Gonyostomum* away from the light, at least, involves a periodic physiological change in the organism—possibly a cessation of photosynthetic activity when sufficient sugar has been formed, such as we have mentioned above, or a reproductive change or both. In fact, it is known that *Gonyostomum* sinks to the bottom and undergoes longitudinal division.

It has been shown by several investigators (see Mast, 1911) that changes in temperature may bring about reversal in the direction of the response to light, but the diurnal changes in the bog pond under consideration do not seem to be important since the decrease in the water temperature as day changes to night is not very great at any one level except at the surface, where, as our studies show, there are ordinarily few individuals of *Gonyostomum.* However, the range of temperatures from surface to bottom is large (Table I) and it is evident that *Gonyostomum* is subjected to rather large changes in water temperature during its movements, so that since a higher temperature often causes green, flagellate forms to react positively to light, the temperature may be a factor which helps to determine the upward migration.

Since we are dealing with an organism which carries on photosynthesis, water temperature must necessarily be an important factor, for it is known that photosynthesis takes place within certain temperature limits. It is surprising to find that during the daytime the congregation of *Gonyostomum* individuals in large numbers along a vertical gradient almost invariably takes place in regions where the temperature is about 24° C. It may be assumed, although not proved, that during the daytime where this congregation in large numbers occurs, photosynthesis is at its height. Numerous observations, not recorded in the text, in which the organisms under discussion were found congregated together during the daytime in large numbers in water of about 24° C., point strongly to temperature as being a factor in determining the vertical distribution, but it does not rule out other factors such as the sunlight, reproductive periodicity, CO_2 content and O_2 content of the water, etc., because we find that during part of the twenty-four hours, namely, the hours of darkness, when photosynthesis has stopped or has been reduced to a minimum, the congregation of *Gonyostomum* individuals in largest numbers does not take place in water of a temperature of 24° C.

There is no intention of offering these results as proof that *Gony-*

ostomum reaches its " optimum " photosynthetic activity at about 24° C., since we have no experimental work to support it and since it is well known that such a so-called " optimum " may vary with the amount, intensity, or quality of other factors concerned in photosynthesis. However, our water temperature records must be taken into consideration in any attempted explanation of the vertical distribution of this organism.

Without having determined experimentally the amount of CO_2 used by *Gonyostomum* in photosynthesis, but judging from the abundance with which this organism occurs at most levels in the pond, it would seem that there is a plentiful supply of CO_2 for their needs from the surface to the bottom. It will be remembered that practically all of this is in the form of free CO_2 and bicarbonate CO_2, both of which can be used by chlorophyll-bearing organisms. The congregation of *Gonyostomum* individuals in rather large numbers at lower levels during the night, when the free CO_2 content of the water may reach 75 p.p.m. or more, indicates that such amounts of free CO_2 are not particularly detrimental to these green flagellates. In fact the decreasing amounts of free CO_2 during the daylight morning hours, from 5 A.M. to 9 A.M., at all levels and the increasing amounts of free CO_2, with one exception, during the dark morning hours, from 1 A.M. to 5 A.M., indicate that photosynthesis takes place at all depths during the daytime. These conditions may be seen in Table I where the total CO_2, free CO_2, and bicarbonate CO_2 are given in parts per million for August 20 and 21, at various depths and times of day.

It must be pointed out that our results are not offered as conclusive proof that photosynthesis takes place at all depths during the daytime. In fact, we know that at times bubbles of gas rise to the surface of the pond which should tend to cause irregularities. However, we think that our results indicate that photosynthesis may occur at all levels.

Since the knowledge of how free CO_2 and bicarbonate CO_2 are used by aquatic, chlorophyll-bearing organisms is quite uncertain and since our studies of the relation between the CO_2 content of the water and photosynthesis have not been experimental in nature, we shall have nothing more to say concerning the relation between the CO_2 content of the water and the vertical distribution of *Gonyostomum* except in so far as to point out that the amount of free CO_2 is one of the factors governing photosynthetic activity.

We can say but little concerning the effect of the oxygen content of the water on the movements of *Gonyostomum.* However, if it can be demonstrated that this organism behaves like the chlorophyll-bearing *Paramecium bursaria* which, according to Engelman (1882) moves toward the light, when there is little oxygen in the water in which it is swimming, so that under the increased intensity the organism will produce more oxygen for respiration as a result of increased photosynthesis, then the small amounts of dissolved oxygen in the lower layers may possibly be considered as a factor in determining the upward migration of *Gonyostomum* individuals during the early morning hours.

Our observations furnish no evidence to support the idea that the organism under consideration migrates vertically due to a search for organic food.

SUMMARY

1. The vertical distribution of the green, euglenoid-like organism, *Gonyostomum semen,* in a bog pond, has been studied at intervals of four hours, throughout twenty-four hours, with reference to air temperature, water temperature, illumination at the surface of the pond, total-, free- and bicarbonate CO_2 in the water, O_2 in the water and the pH value of the water.

2. The practically undisturbed condition of the water in the pond made it possible to demonstrate an oxygen and a carbon dioxide "pulse." These are mainly the result of the photosynthetic activities of *Gonyostomum.*

3. The acidity of this bog pond is partly due to the acid water which enters from the sphagnum bog.

4. A striking chemical characteristic of the pond is the sudden increase in the free CO_2 content of the water between 0.5 and 1.0 meter.

5. Many individuals of the *Gonyostomum* population of this pond move upward during the early morning hours and congregate in maximum numbers somewhere between one meter and the surface. The level at which this occurs seems to be dependent on the proper combination of light, water temperature, and CO_2 content of the water. During the late afternoon and night many individuals move downward so that the maximum counts are found in the lower layers. The upward movement of individuals of *Gonyostomum* from the lower levels is probably due to an increase in the light at the source combined with an increasing gradient of light, temperature, and CO_2 from the bottom to the surface, acting on *Gonyostomum* individuals which have been living in darkness.

The downward movement is probably due to some physiological condition resulting from a sufficient amount of photosynthetic activity or a reproductive tendency or both which is followed by a quiescent sinking.

REFERENCES

Bergman, H. F., 1921. *Am. Jour. Bot.,* **8**: 50.

Brambel, C. E., and R. P. Cowles, 1935. *Anat. Rec.,* **64**, No. 1, Supplement No. 1, Dec. 25: 116.

Chapman, R. N., 1931. Animal Ecology.

Clark, W. M., 1928. The Determination of Hydrogen Ions. Williams and Wilkins. Baltimore.

Clarke, G. L., 1934. *Ecol. Monog.,* **4**: 530.

Cowles, R. P., and C. E. Brambel, 1934. *Anat. Rec.,* **60**, No. 4, and Supplement: 97.

Cowles, R. P., and C. E. Brambel, 1935. *Anat. Rec.,* **64**, No. 1, Supplement No. 1, Dec. 25: 116.

Cowles, R. P., and A. M. Schwitalla, 1923. *Ecology,* **4**: 402.

Dangeard, P.-A., 1889. Le Botaniste, Première Série: 21.

Drouet, F., and A. Cohen, 1935. *Biol. Bull.,* **68**: 422.

Engelmann, T. W., 1882. *Pflüger's Arch.,* **29**: 391.

Esterly, C. O., 1907. *Am. Jour. Physiol.,* **18**: 47.

Esterly, C. O., 1917. *Univ. Calif. Publ. Zoöl.,* **16**: 381.

Esterly, C. O., 1919. *Ibid.,* **19**: 1.

Jennings, H. S., 1906. Behavior of the Lower Organisms, 250–259. Columbia University Press. New York.

Jewell, M. E., and H. W. Brown, 1929. *Ecology,* **10**: 473.

Juday, C., and H. A. Schomer, 1935. *Biol. Bull.,* **69**: 76.

Kikuchi, K., 1930. *Quart. Rev. Biol.,* **5**: 189.

Klebs, G., 1883. *Untersuch. aus dem Bot. Inst. zu Tübigen,* **1**: 311.

Mast, S. O., 1911. Light and the Behavior of Organisms, 272–279. Wiley. New York.

Mast, S. O., 1920. *Anat. Rec.,* **17**: 345.

Muttkowski, R. A., 1918. *Trans. Wis. Acad. Sci.,* **19**, Part 1: 384.

Parker, G. H., 1902. *Bull. U. S. Fish Comm.,* 1901, **21**: 103.

Philip, C. B., 1927. *Ecology,* **8**: 73.

Rose, M., 1925. *Arch. d. Zool. Expér. et Gén.,* **64**: 387.

Russell, F. S., 1927. *Biol. Rev. and Biol. Proc. Camb. Phil. Soc.,* **2**: 213.

Southern, R., and A. C. Gardiner, 1932. *Proc. Roy. Irish Acad.,* **40**: 121.

Utermöhl, H., 1924. *Schrft. f. Süsswasser-u. Meereskunde,* Hft. 9, 11. Jahrg.: 260.

Welch, P. S., 1935. Limnology, 114–115, 94–97, 219–235. McGraw-Hill Book Co. New York.

Whipple, G. C., 1927. Microscopy of Drinking Water, 237–248.

Worthington, E. B., 1931. *Int. Rev. der gesam. Hydrobiol. u. Hydrograph.,* Bd. 25: Hft. 5/6, 394–436.

PROTEIN LIPID BINDING IN PROTOPLASM

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Eleven years ago, Leathes and Raper (1925) wrote: " Clearer ideas as to the physical relation between insoluble fat and the aqueous protoplasmic systems of the living organism, and of the part played by cholesterol in this relationship, are indispensably necessary before we can answer some of the commonest questions in physiological inquiry." The sentence is as true today as when it was written. Although it is now generally realized that protoplasm is more than a solution of proteins, and that non-aqueous lipids are essential to its life, biologists and physiologists have scarcely given thought to the problem of how the lipids are related to the rest of the protoplasm. Most students of the cell are scarcely aware of the existence of the problem. And yet the pathologists have known for many years that the fats and fat-like substances of protoplasm are so bound or united to proteins as to be for the most part non-recognizable in the living or stained cell. It is only when degeneration occurs that the lipids are freed from their union with protein. In such degenerated cells, fats previously concealed may occupy a large part of the cell volume. Obviously, such a freeing of lipids results in an impairment or a loss of protoplasmic activity, and this bears witness to the importance of the lipids for the vital machinery.

The manner in which lipids are bound to proteins in living cells is thus a question which merits investigation, and as will be shown later, it is a question which can be studied experimentally. Unfortunately, however, one difficulty in the way of progress is the lack of definite information as to the manner in which various lipids may unite with proteins in vitro. Thus Handovsky (1933) states: " Die Verbindungen die einzelne Eiweisskörper mit anderen Eiweisskörper oder mit anderen lyophilen Kolloiden (z. B. Lipoiden) eingehen, sind biologisch sehr wichtig, aber wenig exakt untersucht."

The experiments to be reported in this paper were begun as the result of a chance observation. Sea urchin (*Arbacia*) eggs which had been exposed for 4 hours to a solution of oxalated sea water made by adding 1 part of m/4 ammonium oxalate to 2 parts of sea water were centrifuged in an Emerson electric centrifuge (force about 7,000 times gravity). It was then found that the gray cap of these treated eggs was very much larger than that of normal eggs. As is well known, the gray cap of centrifuged *Arbacia* eggs consists of lipid substances. There was thus an apparent increase of the lipid portion of the protoplasm. In the original experiment fertilized eggs were placed in the solution, but later observations showed similar, though not as pronounced effects with unfertilized eggs. With the unfertilized eggs, somewhat longer exposures are necessary.

It was first thought that the action of the oxalated sea water was due to the oxalate ion. However, experiments with potassium oxalate gave no support to this view, and it was realized that the effect produced by the ammonium oxalate was due to the ammonium ion. Indeed, various types of ammonium salts all give essentially the same results. In addition to the oxalate, the acetate, chloride, and sulfate were successfully used.

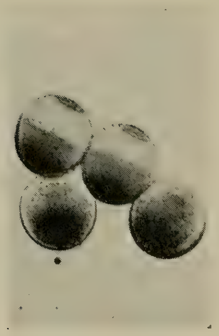
Fig. 1

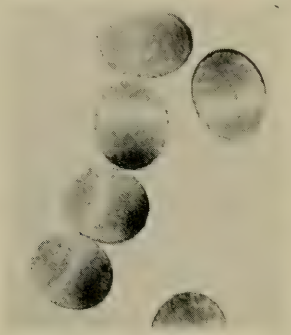
Fig. 2

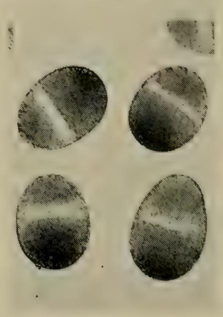
Fig. 3

The details of the experiment are very simple. Eggs are placed in mixtures of sea water and isotonic solutions of an ammonium salt, and are centrifuged after the lapse of a suitable time interval. Solutions of ammonium salts alone can not be used, for they cause a coagulation of the protoplasm, and the eggs then resist stratification on centrifugal treatment. In most of the experiments, one part of an isotonic solution of ammonium chloride was added to 3 or 4 parts of sea water, and eggs were then immersed in this mixture. If unfertilized eggs are used, in 4 or 5 hours, centrifugal treatment shows an increase in the volume of the gray cap. A very marked increase appears after 8 or 10 hours. A typical result is shown in Figs. 1–3. These show eggs which were centrifuged for 3 minutes in an Emerson electric centrifuge at about 7,000 times gravity. The control eggs shown in Fig. 1 were allowed to lie in sea water for 8 hours before being centrifuged; they show a typical small gray cap. Figs. 2 and 3 show the appearance of eggs centrifuged after exposure to 70 parts sea water plus 30 parts 0.53 m NH_4Cl for 8 hours. The centrifugal treatment of the experimental eggs was the same as that of the control, but the eggs exposed to the ammonium salt show a greatly enlarged gray cap. Indeed the gray cap is so large that the hyaline zone is reduced to a narrow streak.

It might be thought that in these eggs with enlarged gray cap, the increase in volume is due to a greater scattering of the materials of the cap. However, when the eggs are centrifuged for short or long periods, the volume of the gray cap remains essentially the same. Such centrifugal treatment can not be continued indefinitely, for the eggs eventually break into two; but with the most vigorous centrifugal treatment possible, the gray cap of the eggs exposed to ammonium salts is always much larger than that of normal eggs.

The behavior of *Arbacia* eggs toward solutions of ammonium salts is not peculiar to this type of material. When eggs of the clam *Cumingia* are exposed to mixtures of sea water and isotonic ammonium chloride or oxalate, there is in this case also an increase in the width of the fatty zone when the eggs are centrifuged. This increase is not as striking as in the case of *Arbacia*. In *Cumingia* eggs, the normal gray cap or zone of fatty materials appears rather dark under the microscope. What appears to be the added accumulation after treatment with ammonium salts is somewhat lighter or more transparent. A similar distinction can sometimes be observed in *Arbacia* eggs.

Ammonium salts may also cause an increase in the visible fat of *Amœba proteus*. In experiments performed in May, 1935, amœbæ were kept in dilute solutions of ammonium chloride for 3 or 4 hours and were then centrifuged vigorously (5 minutes at 7,000 times gravity). The solutions used were m/60 or m/120 NH_4Cl, at a pH of approximately 6.0. In one case, 1 part of m/60 NH_4Cl was added to 2 parts of m/40 NaCl (pH 6.5). Numerous experiments were tried. In every case, care was taken to select cultures which normally had no free fat or lipid in them, as occasionally cultures are found which contain free fat even without treatment. Normally, when an *Amœba proteus* is centrifuged vigorously, the crystals of the protoplasm accumulate in the heavy or centrifugal half of the cell and the rest of the cell is clear and transparent (see Heilbrunn and Daugherty, 1932). However, after treatment with ammonium salts, the centrifuged amœbæ had approximately a fourth or more of the cell filled with particles lighter than the rest of the fluid protoplasm. These light particles stained brilliantly with Sudan III and were thus clearly lipoid.

Amœbæ constitute a much more variable material than *Arbacia* eggs.

It has already been noted that occasional cultures show free fat even without treatment with ammonium salts. On the other hand, in experiments done in March, 1936, no free fat could be demonstrated even after treatment with ammonium salts. However, in June, 1936, experiments with ammonium chloride were successful in 80 per cent of the amœbæ tested. Perhaps there is a seasonal variation. Whether this is true or not, one can distinguish three types of amœbæ. In the first type, free fat is present normally; in the second, free fat appears only after treatment with ammonium salts; and finally there are amœbæ in which there is no free fat even after exposure to ammonium salts.

Similar variations appear to occur in frog muscle. Students in my laboratory have studied the effect of ammonium salts on the detectable fat in frog muscle fibers (*Rana pipiens*). Mr. H. Blumenthal exposed gastrocnemius muscle to isotonic solutions of ammonium salts mixed with Ringer's solution, and he found on fixing in osmic acid solutions that there was a great increase in the free fat. Similar results were also obtained with heart muscle of the frog. Mr. Newman has repeated Blumenthal's experiments using single muscle fiber preparations from the frog sartorius, and staining with Sudan III. In the winter, the normal muscle fibers are without free fat, but show a large amount after exposure to ammonium salts. In the spring, the normal muscle fibers have free fat even before treatment. In the late summer, it is possible that free fat may be absent even after ammonium treatment. This would accord with the observation of Bell (1911) that during the summer months it is not ordinarily possible to demonstrate fat-staining granules in *Rana pipiens* muscle, although such granules are easy to observe during the spring and fall.

It is a remarkable fact that in various types of protoplasm, in sea urchin eggs, in clam eggs, in amœba, and in frog muscle, treatment with ammonium salts results in an increase in the free fat or lipid. In the sea urchin egg, this increase in fatty materials is indicated by a very marked increase in the volume of granules lighter than the rest of the protoplasm. That these lighter granules are fatty seems reasonably certain. They could scarcely be protein, for proteins constitute the heaviest rather than the lightest constituents of the protoplasm. An attempt was made to stain the lighter granules of the sea urchin egg with Sudan III. This did not prove very satisfactory, perhaps because of the small size of the granules. In amœba, on the other hand, the light granules set free following treatment with ammonium salts stained very readily with Sudan III. So, too, in the muscle experiments, fat could be demonstrated both by osmic acid and by Sudan III.

It might be argued that in the experiments with *Arbacia* eggs, the increase in volume of the oil cap was due to an increased affinity of the oil for water. However, if *Arbacia* eggs are broken into two fragments by very vigorous centrifuging, and the heavier fragments which are free from fat or oil are then treated with ammonium salts, there is an accumulation of lighter particles at the centripetal pole of the heavy fragments when these are centrifuged. This indicates that the lipid material which appears following ammonium salt treatment can originate from protoplasm freed from its original fatty substances. Moreover, it may be noted again that in amœba, cells totally without free fat may be made to show a considerable quantity after treatment with ammonium salts.

How can one account for the increase in lipid materials? Although change from protein to fat is possible, it can readily be shown that no such transformation is responsible for the results outlined above. By gathering large masses of eggs, it was possible to make quantitative determinations of the total lipid content of normal eggs as compared with the lipid content of eggs treated with ammonium salts. In order to insure an effect of the ammonium salts, the eggs must be placed in large flat dishes so as to avoid crowding during the experiment. A concentrated egg suspension obtained from a hundred or more sea urchins was divided into two equal portions. One portion was allowed to remain in sea water. To the other portion, 0.53 m NH_4Cl was added in the proportion of 3 parts of the solution to 7 parts of sea water. After 8 hours, the eggs from each portion were collected and analyzed for total lipid. In one experiment, extraction was performed with a modified Soxhlet apparatus, in a second the wet material was extracted with alcohol and ether. Both tests showed almost identical quantities of total lipid in eggs treated with ammonium salt and controls. These tests were performed with the assistance of Mr. Samuel Koppelman.

As a result of the chemical analyses, it may be concluded that the action of ammonium salts is to free bound lipid from union with protein. That living cells normally contain a relatively large amount of fat or lipid in the bound state is abundantly clear from the literature on fatty degeneration. Cells may appear to be practically free from fatty substances, whereas upon degeneration they become filled with numerous fatty droplets. And yet, often in these cells which have undergone fatty degeneration, no increase in total lipid can be demonstrated by chemical methods. Thus, obviously, bound fats or lipids occur in protoplasm. In blood, also, the lipids are to a large extent bound to the proteins.

The mechanism of action of the ammonium salts is to some extent clear. Jacobs (1922) has shown that when cells are exposed to a medium containing ammonium salts, the pH of the protoplasm rises. This is due to a hydrolysis of ammonium salt to ammonium hydroxide and acid. The ammonia or ammonium hydroxide is then able to penetrate the cell, whereas the inorganic acid remains outside.

That the effect of the ammonium salts is in reality due to an alkalinization of the protoplasm is supported by two sets of observations. In the first place, ammonium hydroxide also causes an increase in free or visible lipid. Thus sea urchin eggs were exposed for several hours to solutions with enough ammonium hydroxide added to bring the pH to 8.9 in one case, and to 9.2 in another. To avoid excessive precipitation of magnesium hydroxide, the ammonium hydroxide was not added to sea water, but to a sodium chloride-calcium chloride mixture (125 cc. 0.53 m NaCl + 1.5 cc. 0.3 m $CaCl_2$). When the eggs were centrifuged after exposure to these solutions, there was a marked increase in the volume of the oil cap, and the appearance of the eggs was like that shown in Figs. 2 and 3.

In the second place, it was discovered that an excessive amount of carbon dioxide in the medium surrounding the cells prevented an increase in free fat or lipid. Thus when egg cells were crowded, even though they were in contact with the proper strength of ammonium chloride, no increase in visible fat occurred. Also when carbon dioxide was bubbled through the medium, the action of the ammonium chloride was inhibited. Carbon dioxide readily penetrates cells and it doubtless tended to prevent the alkalinization of the protoplasm which might otherwise have been caused by the ammonia diffusing into the cells from the ammonium chloride solution.

It is quite conceivable that alkalinization in itself might cause a liberation of lipid from protein-lipid combination. Thus Theorell (1930), who has done some of the most interesting work in the little-studied field of protein-lipid binding, states:

"In der Literatur findet man mehrere experimentell mehr oder weniger wohlbegründete Angaben, das sowohl Cholesterin als auch Phosphatide an die Globuline, besonders an das Euglobulin, gebunden seien. Gäbe es eine solche Verbindung, wäre es ja nicht unwahrscheinlich, dass sie bei dem isoelektrischen Punkte der Globuline dissoziiert wäre, wo ja ihr Minimum an elektrischer Ladung und Hydratation zu finden ist."

Theorell himself studied the binding of globulin and cholesterol and found that although the cholesterol was bound at either side of the isoelectric point, at the isoelectric point itself there was no binding. However, in the case of albumin the situation is not quite so simple, and a recent paper of von Pryzlecki, Hofer, and Frajberger-Grynberg (1935) indicates the complexities of protein-lipid binding.

In protoplasm, it is quite possible that increase in pH does bring a shift toward the isoelectric point, of at least certain of the protein constituents. In this connection, it is interesting to remember that there is evidence that protoplasmic particles migrate to the cathode when an electric current passes through a cell (see Heilbrunn, 1928). This would indicate that there are proteins present which are on the acid side of the isoelectric point.

SUMMARY

1. When various types of protoplasm are treated with dilute solutions of ammonium salts, there is an increase in the free fat or lipid.

2. This effect is due to an alkalinization of the protoplasm.

LITERATURE CITED

Bell, E. T., 1911. The interstitial granules of striated muscle and their relation to nutrition. *Intern. Monatschr. f. Anat. u. Physiol.*, **28**: 297.

Handovsky, H., 1933. Allgemeine Chemie der Proteine. In Oppenheimer's Handbuch d. Biochemie d. Menschen u. Tiere. Second edition. Ergänzungswerk, vol. 1, p. 320.

Heilbrunn, L. V., 1928. Colloid Chemistry of Protoplasm. Berlin. G. Borntraeger. 1928.

Heilbrunn, L. V., and K. Daugherty, 1932. The action of sodium, potassium, calcium, and magnesium ions on the plasmagel of Amœba proteus. *Physiol. Zoöl.*, **5**: 254.

Jacobs, M. H., 1922. The influence of ammonium salts on cell reaction. *Jour. Gen. Physiol.*, **5**: 181.

Leathes, J. B., and H. S. Raper, 1925. The Fats. Longmans, Green and Co. Second edition. London, 1925.

Przylecki, S. J. von, E. Hofer, and S. Frajberger-Grynberg, 1935. Über Affinitäten zwischen Proteinen und Fettsäuren, Fetten, oder Lipoiden. *Biochem. Zeitschr.*, **282**: 362.

Theorell, H., 1930. Studien über die Plasmalipoide des Blutes. *Biochem. Zeitschr.*, **223**: 1.

FREE CALCIUM IN THE ACTION OF STIMULATING AGENTS ON ELODEA CELLS

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INTRODUCTION

A number of investigations have been made in this laboratory on the colloid-chemical changes in the protoplasm that are involved in cell stimulation. The results conform to the hypothesis, discussed most fully by Heilbrunn and Daugherty (1933), that a primary effect of stimulation is the release of Ca ion in the cell. Heilbrunn, Mazia, and Steinbach (1934) (fuller accounts to be published soon) reported that direct analysis after ultrafiltration showed that in the fertilization of sea-urchin eggs and in the injury of muscle, the free Ca content of the cells indeed increases. On the other hand, changes which are interpreted as evidence of Ca release are prevented by oxalates (Heilbrunn and Young, 1930; Heilbrunn and Daugherty, 1933).

At the present stage of the problem, a qualitative, intracellular method would be even more valuable than our quantitative ultrafiltration method for a general testing of the hypothesis of Ca release. The chemical accuracy of the latter method is offset by the fact that masses of cells must be used, and must be subjected to violent means of killing. An intracellular method in which it is not necessary to add reagents is ideal. Just such a method is provided in the present case by plant cells which contain high concentrations of soluble oxalates in their sap. An increase in the free calcium concentration of the cell is immediately indicated by the formation of calcium oxalate crystals.

Although there is a large literature on the natural occurrence of calcium oxalate, dating back to Malpighi (1675) (*cf.* Patschovsky, 1920), there are very few papers on its experimental production in cells in which it does not normally occur. Osterhout (1910) used crystal formation as a test of Ca penetration into root hairs of *Dianthus*.

The use of the cells of *Elodea,* in which crystals do not occur normally, was suggested to us by the work of Nadson and Rochline-Gleichgewicht (1927) and Rochline-Gleichgewicht (1930). These authors noted the appearance of CaC_2O_4 crystals in cells of *E. densa, E. canadensis,* and *Pterygophyllum hepaticæfolium* after ultraviolet irradiation, after exposure to radium emanation, and after plasmolysis.

Identification of Crystals

Calcium oxalate was produced, in our experiments, as tetragonal crystals of the trihydrate and the monoclinic crystals of the monohydrate. The former are the most characteristic, as well as the largest and most easily recognized. Large crystals of both types are shown in Fig. 1. In addition to such crystals, sometimes small, needle-like crystals of calcium oxalate are formed. Their habit cannot be determined.

Although such appearances correspond to descriptions of calcium oxalate in the literature, and to our own *in vitro* products, it seemed worth while to verify this. The solubilities of experimentally produced crystals in 5N H_2SO_4 and in 10M acetic acid were compared. The crystals dissolved in the sulphuric, but not in the acetic acid. This behavior is characteristic of calcium oxalate. But the most elegant method for the identification of microscopic crystals is that of refractive index determination.

Such determinations were made for us by Mr. A. W. Postel, of the Geology Department of this University. They demanded the greatest skill and patience, as the largest crystals obtainable in *Elodea* cells are much smaller than those usually found in petrological material.

The indices of refraction were obtained by the immersion method. The crystals were bathed in liquids of known refractive index, until those liquids were found which had the same indices as the crystals; in such liquids all relief between crystal and liquid vanishes. In the present work the Becke line method was used (for description see Chamot and Mason, 1931, Vol. 1). Since the crystals in our cells were anisotropic, the measurements had to be made with the polarizing microscope, as the values of the several indices of the same crystal can be obtained only by placing the vibration planes of the crystal successively in a position parallel to the vibration plane of the lower Nicol prism.

The largest crystals were obtained in leaves plasmolyzed for about thirty minutes in 0.5M sucrose, and deplasmolyzed in tap water. These leaves were dehydrated in ethyl followed by isoamyl alcohol.

The matching liquids were a series of mixtures of isoamyl alcohol and alpha-monochlor-naphthalene (halowax oil), ranging in refractive index from 1.440 to 1.600 in gradations of about 0.005. The liquids were introduced into the dehydrated cell by the " sinking method " used for clearing microscopic preparations (Lee, 1900, p. 79). After the leaf had sunk through a column of a mixture, it was immersed for some time in a fresh quantity of the same liquid. Five-cc. samples were used.

The leaf was now ready for examination with the polarizing microscope. When matches were obtained, the exact refractive indices of the liquids were determined by means of a goniometer with a hollow prism for the liquid.

Exact values were obtained for the tetragonal crystals. The indices that could be measured came out as follows:

ω	ϵ
1.491	1.540
1.505	
1.499	
1.495	

These figures are in agreement with those given by Wherry and Keenan (1923). The shape of the other crystals, as well as the fact that they had one index of refraction higher and one lower than the highest index of the tetragonal crystals, was taken as sufficient evidence that they were monoclinic crystals of calcium oxalate monohydrate.

The optical properties of our crystals, therefore, identify them as calcium oxalate. Parallel determinations on artificially prepared calcium oxalate monohydrate and trihydrate verified this conclusion.

ACTION OF STIMULATING AGENTS

Agents Used

Stimulating agents may be defined as physical or chemical agents which call forth the characteristic responses of irritable cells. In working on a general theory of stimulation, it is necessary to use those agents which affect the most familiar types of responsive cells, such as muscle, nerve, and unfertilized egg cells. Among such general stimulants are electric shocks, radiations, changes in temperature, mechanical shock, a variety of chemical agents, and osmotic changes. To test Heilbrunn's hypothesis, which was the purpose of this work, cells of *Elodea canadensis* were subjected to all these influences to see whether the latter caused a release of calcium in the cell interior.

EXPLANATION OF PLATE I

FIG. 1. Crystals produced by plasmolysis in 0.5M sucrose and deplasmolysis in pond water. Crystals at lower left tetragonal, others monoclinic.

FIG. 2. Crystals produced by two condenser shocks. Twenty volts, 2 microfarads.

FIG. 3. Deposit of crystals toward anode (left in all figures). Current 0.75 milliampere for 15 seconds.

FIG. 4. Deposit of crystals toward anode, under higher magnification. Same current as in Fig. 2.

FIG. 5. Electrical breakdown of plasmolyzed cells. Normal cell marked by arrow.

FIG. 6. Same field at lower focus, showing crystals which have settled down within the tonoplasts.

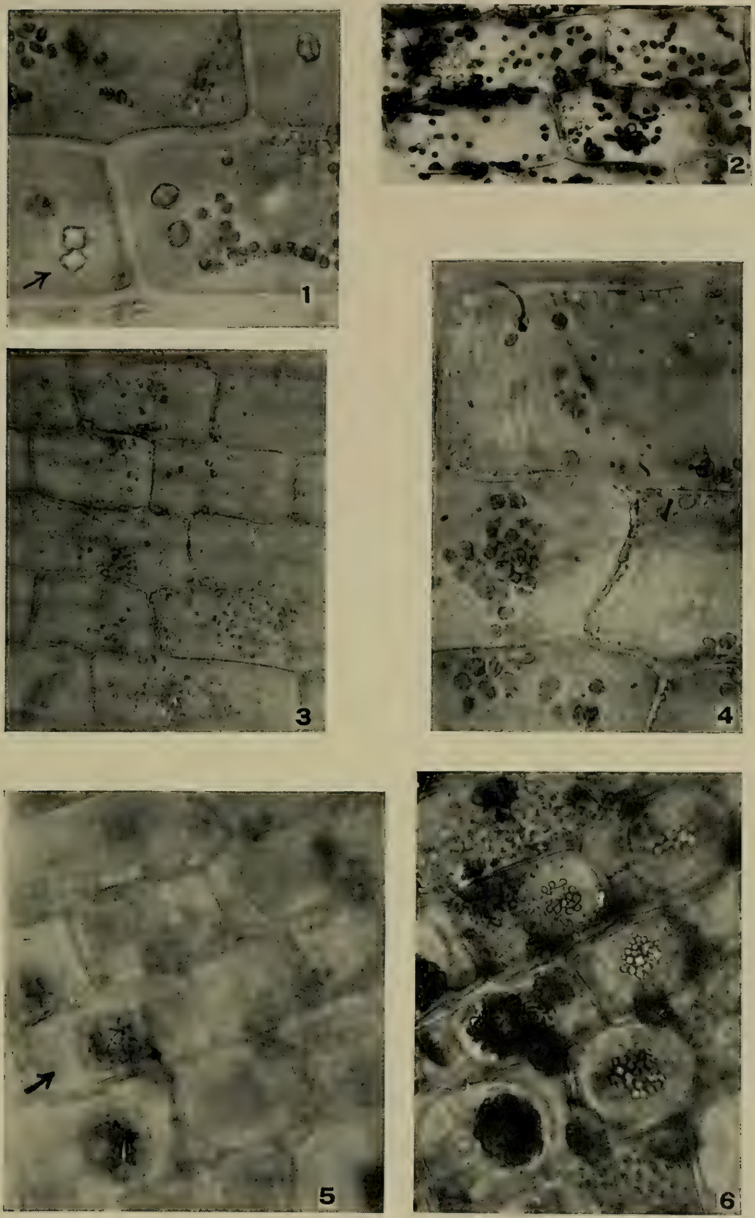

Plate I

Effect of Electric Current

Condenser Discharges.—In studying electrical stimulation it seemed desirable to use shocks of brief duration in order to minimize such factors as electroendosmosis. A condenser of 2-microfarad capacity was charged from a " B " battery that gave about 20 volts, and discharged through Cu-$CuSO_4$ electrodes of simple design. Glass tubes of 6 mm. bore were filled at their tips with a 3–4 per cent agar gel made up in tap water or 0.1M NaCl. Over the gel was placed saturated $CuSO_4$ solution, and the copper wires were dipped into this.

The *Elodea* leaf was dried on filter paper and placed on a glass slide. The electrode tips were placed a few millimeters apart, because the resistivity of the tissue was found to be so great that effective currents could not be passed through the whole length of the leaf with the apparatus used. When more than one shock was used, the shocks were spaced at intervals of one second or two seconds.

At first, 5–10 shocks were used. These caused the deposit of many crystals in the vacuoles although the protoplasm streamed vigorously, more vigorously if anything, after the shocks. It was soon found that definite production of crystals results from application of a single shock, or two shocks. Cells that had received two shocks are shown in Fig. 2. In general, more shocks produced more crystals. One had to use more than 20 shocks to kill some of the cells.

The crystals were localized at the anodal end of the cell, as Fig. 2 shows. Later they became scattered as a result of streaming motion, but if observed immediately after stimulation, it could clearly be seen that calcium oxalate forms at the anodal end on stimulation.

Continuous Direct Current.—When a 110-volt source of D.C. was used, the current could be passed through the whole leaf, and the cells observed during the passage of the current. The same electrodes were used as in the condenser experiments.

When a current of 1 milliampere is made and instantly broken, the immediate effects are a slight but definite jerking of the protoplasm away from the cathodal end of the cell, and the appearance, very soon, of small crystals of calcium oxalate in the vacuole at the anodal end. If the current is allowed to flow for a second or two, and then broken, the same effects become more pronounced. At the cathodal end the protoplasmic layer thickens markedly and the convex contour of the tonoplast becomes visible. At the anode the protoplasmic layer becomes thin, and the protoplasmic strands in the vacuole are broken, the protoplasm being drawn into the main mass. More crystals appear in the vacuole at the anodal end. If at this stage the cells are treated with 0.33M sucrose, they plasmolyze, but the protoplasts adhere to the cell wall at the anodal end.

If a larger amount of current is used, a layer of small crystals forms along the extreme anodal boundary of the protoplast, appearing to be contiguous to the cell wall (Figs. 3, 4). A latent period may intervene between the breaking of the current and the appearance of this crystal deposit. The formation of this anodal deposit of crystals marks the beginning of the disintegration of the cell membrane. This first appears toward the anode. With further passage of the current, the disintegration passes in a wave over the surface of the protoplast, from anode to cathode. Occasionally, it has seemed that it went in the reverse direction. As the wave passes, calcium is released. Apparently free oxalate is available at the site of the disintegration, for the wave leaves behind it a wake of calcium oxalate crystals on the surface of the protoplast. The wave passes rather slowly, its speed depending on the current applied. If the current is broken, it stops, and is seen as a tenuous, refractive boundary, the edge, apparently, of a perfectly transparent layer.

The wave reported by Weis (1925) in *Allium cepa* cells may be similar, though from his description we would judge that he was observing a cathodal movement of the tonoplast.

Another phenomenon observed in cells subjected to severe currents was the frequent pulling-in of the protoplast along the sides of the cells, particularly at points where it was convex.

Once a deposit of crystals has formed along the anodal end of the protoplast, it is no longer possible to plasmolyze the cells; they do not recover their semipermeability on standing. The chloroplasts shrink and assume puckered shapes.

To determine the relationship between membrane disintegration and calcium release, cells were plasmolyzed with 0.5M sucrose, and the protoplasts were subjected to the electric current in this medium. Since the conductance is low, most of the current is shunted through the cells, so that small currents will have severe effects.

Currents of 0.5–1 milliampere were used. When the circuit is made and instantly broken, all the protoplasts jerk toward the anode (*cf.* Rubinstein and Uspenskaya, 1934). Slightly more current causes some cells to break down; others are more resistant, and some never break down.

The cells which do break down rupture first at the anode. The plasmalemma breaks, at one point as a rule, but sometimes at several. Protoplasmic granules flow out, and a few calcium oxalate crystals are seen to form outside the protoplast. The tonoplast protrudes through the point of rupture, enlarging the break in the protoplasmic layer. The clear contents of the vacuole become filled with crystals, beginning at the point of exposure, and a wave of plasmalemma disintegration passes over the surface from anode to cathode, leaving the tonoplast to round up again (Figs. 5, 6). The protoplasmic layer, also broken at the anode, "peels" off from the tonoplast, and is clumped at the cathodal end of the cell. Sometimes the tonoplast is freed in two parts, or fragments break away, becoming small, clear spheres containing crystals.

When the current is allowed to flow for 15–30 seconds, the tonoplasts, too, break down, contracting first, then disintegrating after anodal rupture.

Our experiments did not reveal any natural polarity in the cells of the *Elodea* leaf. The results were the same whether the current was passed from base to tip or vice-versa. If the leaf was placed transversely in the electrical field, crystals were in this case also deposited at the anodal end.

The explanation of the anodal calcium release cannot be discussed at length here. An obvious suggestion is that a local increase in acidity decreases the capacity of the proteins to bind Ca. However, the observations of Kühne (1864), Bethe (1915), Mast (1931), led to the conclusion that plant cells subjected to direct currents become more alkaline at the anode, and acid at the cathode. However, Blinks (1932) attributes the apparent polar change in color in the natural anthocyanin indicators used by the above authors to electrophoresis rather than to change in reaction.

We performed a number of experiments to see whether acid could cause the setting free of Ca and the precipitation of calcium oxalate. Leaves were placed in 0.1N or 0.2N acetate buffers of pH 3.8, 4.2, 4.6, 5.0, 5.8. These external pH values tell nothing about the effect on the intracellular pH, for the acetic acid penetrates rapidly while the sodium acetate penetrates slowly if at all. The leaves in these solutions were examined at intervals up to two hours. Various morphological changes were observed, but in no case did crystals appear. Therefore, the hypothesis that local acid accumulation is responsible for calcium release when direct currents are applied was not verified.

Effect of Oxalate.—The vacuole of the *Elodea* cell contains a fairly high concentration of free oxalate. There is also reason for believing that there is oxalate in the protoplasm (see discussion). Yet, if the observations of Heilbrunn and Daugherty (1933) are of general significance, we must expect that immersing the *Elodea* cell as a whole in an oxalate solution should prevent calcium release. In testing this, leaves were washed in 0.05M sodium oxalate before they were exposed to electric currents. Since this solution has such a high conductivity as compared to the normal medium of the cells, they were removed from it after a minimum of ten minutes exposure and placed in 0.001M sodium oxalate or in distilled water. The same strengths of electric current were applied to these cells as had been applied to cells not washed in oxalate.

The results were consistent in many experiments. No crystals were produced no matter how long the current was passed. The plasmalemma did disintegrate in a wave beginning at the anode, but even this was not accompanied by crystal production.

Mechanical Stimulation

Mechanical shock is a very general stimulant. Its effects on cells are discussed in a review by Lepeschkin (1936) and by Angerer (1936). The latter, working on *Amœba,* has found that the viscosity changes associated with mechanical agitation are similar to those described by Heilbrunn and Daugherty (1933) for ultraviolet irradiation, and therefore in accord with the hypothesis that calcium release is a primary effect of the stimulation.

Our method of applying the stimulus was very primitive. A leaf was placed between two microscope slides and rapped sharply with heavy forceps. In one experiment in which the leaf was rapped 50 times and examined immediately, more than half of the cells showed very clear crystal formation, most of the crystals being of the needle-like variety. The cells were not killed, but seemed to be streaming at a somewhat more rapid rate than normally. The effect of mechanical stimulation was tried on leaves from many different specimens of *Elodea.* Crystals always could be obtained, although sometimes very many shocks were necessary.

Irradiation with Ultraviolet Light

The valuable paper of Nadson and Rochline-Gleichgewicht on the production of calcium oxalate crystals as a result of ultraviolet irradiation has already been mentioned. There was no difficulty in verifying the observations of these authors. Leaves were irradiated from a Cooper-Hewitt quartz mercury vapor lamp, at a distance from the arc of 25 centimeters. The leaves were exposed for 2.5 minutes, surrounded only by an adhering film of water. On examination within five minutes after treatment, they showed a definite but not profuse precipitation of calcium oxalate in the vacuoles of the cells. Most of the crystals were tetragonal. Three minutes irradiation caused a much heavier precipitation.

At the same time leaves were irradiated after having been immersed for 15 minutes in 0.05M sodium oxalate. In these leaves even 10 minutes after irradiation there was no sign of calcium oxalate in the vacuoles of the cells.

Therefore, just as in the case of electrical stimulation, treatment of the outside of the cell with oxalate prevents the release of calcium ion in the interior.

Heat

Heat stimulates many living systems; it is listed by Bethe (1915) among the general protoplasmic stimulants. In our preliminary experiments *Elodea* leaves were immersed in water at 100° C. This treatment killed the cells without producing any crystals.

We then tried heat treatments of the same order of magnitude that have been found to stimulate other cells, particularly egg cells. Heating at 35° for more than an hour caused no crystal production. At 40° many crystals, a mixture of fine needles and large tetragonal crystals, were formed after 60 minutes exposure. At 45° exposures of 20 minutes duration produced many crystals in all the cells. These cells plasmolyzed normally, after the heat treatment, in 0.3M sucrose. At 50°, 8 to 10 minutes exposure caused the production of crystals without destroying the ability of the cells to plasmolyse in the 0.3M sucrose solutions. At 60°, however, all the cells died after exposure of a minute or two—and no crystals were produced!

To determine the effect of oxalate on the reaction of the cells to heat, leaves were washed in 0.05M sodium oxalate, and subjected to the heat treatments described above while they were still in the sodium oxalate solution. No crystals were produced.

We attempted to decide whether the effects described above were due to the absolute heat treatments we used, or whether temperature change was the causal factor. We studied the effect of rapid cooling by immersion in an ice-water mixture. No crystals were produced by 10-, 20- and 60-minute immersions. When the cooled cells were placed in water at room temperature, no crystals were produced. Therefore, high temperatures themselves were responsible for crystal production by heating.

Osmotic Changes

Bethe, in the list of general stimulants cited above, includes osmotic influences; hypertonic solutions particularly are stimulating agents.

If cells are plasmolyzed in 0.5M sucrose and deplasmolyzed in pond water, they are then found to have crystals in their vacuoles. When the process is followed under the microscope, it is observed that the crystals do not appear until deplasmolysis begins. The first effect observed, upon addition of the deplasmolyzing agent, is a strong contraction of the tonoplast. This is observed, even after complete, convex plasmolysis. It is strikingly rapid and vigorous, and even after harsh plasmolysis in 1.3M $CaCl_2$ deplasmolysis is preceded by further contraction.

After strong plasmolysis, e.g., after 10 minutes or more in 0.5M sucrose, deplasmolysis leads to the death of most of the cells of *Elodea* leaves. The protoplasm expands in a series of slight jerks, which are due to the rupture, at various points, of the plasmalemma. The vacuoles of such cells acquire many crystals, even when a deplasmolyzing agent such as 0.25M sucrose or distilled water, lacking any calcium, is used. In this type of fatal plasmolysis, the tonoplast breaks down as well as the plasmalemma.

When weaker plasmolysis is used, such as follows exposure for less than 10 minutes to 0.5M sucrose, or 10–15 minutes in 0.33M sucrose, deplasmolysis is smooth and the cell may be replasmolyzed. Less crystals are obtained in these cells, but there is always a definite precipitate. Sometimes these crystals may adhere to the vacuole wall and be carried about as the protoplasm streams. When such cells are bathed in 0.05M $CaCl_2$, no further precipitation of calcium oxalate is observed.

Many experiments on the effects of very weak plasmolysis were performed. When cells are treated with a 0.2M NaCl solution, there is a preliminary stage, lasting about three minutes, in which the vacuole shrinks markedly, but the plasmalemma does not separate from the cell wall. If a hypotonic solution is added as soon as this preliminary stage of plasmolysis is established, the tonoplast expands and the cell resumes its normal appearance without the production of any crystals. If, however, 2–3 minutes are allowed to elapse before the 0.2M NaCl is changed for pond water or any other hypotonic medium (the exchange is effected under the microscope simply by drawing the new medium under the coverslip with strips of filter paper), the protoplast, which had not yet contracted in the hypertonic medium, separates from the cell wall, and within a few seconds expands again. In cells in which this transient plasmolysis occurs, crystals are formed. It seems, therefore, that actual shrinkage of the protoplast is necessary to cause calcium release after treatment with hypertonic solutions. It is immaterial whether this plasmolysis is produced while the cell is in the hypertonic medium, or as a result of the "contraction" that occurs on transfer from the hypertonic medium to the hypotonic.

Other experiments on the effect of mild plasmolysis were performed with the balanced mixture (0.18M NaCl plus 0.005M $CaCl_2$) as plasmolyzing agent, and the same mixture diluted to half strength as deplasmolyzing agent. The time of exposure to the hypertonic solution was varied. The cells showed vigorous protoplasmic streaming after deplasmolysis, and the vacuoles contained crystals, usually small ones but sometimes numerous. We were unable to correlate time of plasmolysis with the amount of precipitate, although a quantitative method might reveal such a correlation. The precipitate could not be increased by washing the cells with a 0.05M $CaCl_2$ solution, except in those few cells that had been broken even by the mild treatment.

These experiments answer the objection that the crystal formation may be ascribed to penetration of calcium from the external medium, after rupture of the plasmalemma by our treatment. First, in the experiments with mild plasmolysis there is no indication of membrane rupture. Second, although after strong plasmolysis more crystals can be obtained when 0.05M $CaCl_2$ is used as deplasmolyzing agent, very many crystals are obtained when distilled water, 0.1M NaCl, or 0.25M sucrose are used. Finally, when 0.05M sodium oxalate was used as deplasmolyzing agent, fewer crystals were produced, but there was a definite, sometimes an abundant, precipitate.

The effect of oxalate on the calcium release in plasmolysis-deplasmolysis was investigated. Cells were immersed for various periods in a solution of 0.5M sucrose in 0.1M sodium oxalate. With prolonged immersion—30 minutes or more—the cells gradually undergo " systrophy," in which the main mass of cytoplasm and cytoplasmic inclusions flows together at one part of the cell, while the remainder appears as a thin layer around the now-visible tonoplast (*cf.* Küster, 1929, p. 172). If the cells at any time after plasmolysis in this solution are treated with distilled water, they deplasmolyze. Typically no crystals are produced.

In this part of the work two types of experiments are to be distinguished: (1) experiments in which plasmolysis is brief, or in which the weakly hypertonic solution 0.33M sucrose in 0.1M $Na_2C_2O_4$ is used; and (2) experiments in which strong plasmolysis, leading to systrophy, is obtained in 0.5M sucrose in 0.1M sodium oxalate. In the first group of experiments, deplasmolysis was smooth and the cells were not killed; they could be replasmolyzed. A number of deplasmolyzing agents were tried: distilled water, pond water, 0.1M NaCl plus 0.005M $CaCl_2$, 0.05M sodium oxalate. No crystals were obtained with any of these. Therefore the results are in accord with those obtained with electrical, thermal, ultraviolet stimulation: washing in oxalate prevents the release of calcium which otherwise takes place.

In the second group of experiments, in which deplasmolysis of the systrophied cells does involve rupture, no crystals were obtained when distilled water or 0.05M sodium oxalate was used, but crystals were obtained when pond water or 0.005M $CaCl_2$ was used. This result is enlightening, for it shows that the tonoplast is permeable to Ca; observation showed that the crystals were formed as soon as the plasmalemma ruptured.

To test whether the oxalate actually removes the Ca from the surface, or whether the presence of the oxalate ion itself is responsible for the prevention of crystal formation, some cells were first plasmolyzed in 0.5M sucrose in 0.1M sodium oxalate, then washed in 0.5M sucrose alone, then deplasmolyzed in 0.25M sucrose or in distilled water. No crystals were produced. Therefore, the oxalate does remove the Ca or whatever is responsible for the Ca release, from the surface.

If it is Ca that is removed from the surface by oxalate, one might be able to restore it, and thus to reverse the effect of the oxalate. Leaves treated with 0.05M sodium oxalate for 10 minutes—more than enough to prevent crystal formation, were washed in distilled water. Some of them were then placed for ten minutes in pond water or 0.02 $CaCl_2$, others not. Both groups were then plasmolyzed in 0.05M sucrose and deplasmolyzed in distilled water. The cells that had been treated with pond water or $CaCl_2$ formed crystals on deplasmolysis—the effect of the oxalate had been reversed. The other leaves showed no crystals, or many fewer crystals. Therefore, oxalate does indeed act by removing Ca from the surface, and this Ca may easily be replaced.

One interesting observation made in this part of the work was that violent deplasmolysis after plasmolysis in oxalate-containing solutions did not destroy the tonoplast. Especially after systrophy, completely isolated tonoplasts could be obtained.

Substitution of Citrate for Oxalate

In the experiments on the prevention of calcium release, oxalate was used because it had been used by the previous authors whose ideas we were testing. Similar experiments were later performed in which citrate was substituted for oxalate. Although citrate does not precipitate Ca, it binds it in a non-ionized complex. Furthermore, the pK of this $CaCit^-$ is so large, that at high citrate concentrations the citrate should bind Ca at the expense of proteins (*cf.* McLean and Hastings, 1935).

Our tests were made with electrical stimulation, and with plasmolysis-deplasmolysis treatment. Leaves were washed in 0.04M sodium citrate for 10 minutes, then rinsed in distilled water and subjected to direct currents of about 1 milliampere. No crystals were formed, although the usual deposits were obtained in cells treated with distilled water alone.

The only visible effect of immersion in sodium citrate was a tendency of the chloroplasts to flow together, which was reversed on their return to pond water.

In studying the effect of citrate on plasmolysis-deplasmolysis, the following procedure was used. Leaves were washed in 0.035M sodium citrate for 10 minutes, then rinsed in distilled water and plasmolyzed in 0.05M sucrose. On deplasmolysis with distilled water no crystals were formed. In control leaves, treated similarly except for the exposure to citrate, the usual precipitate of calcium oxalate was obtained. It must be remarked here that this experiment is much clearer than the corresponding one with oxalate; in the latter there are occasional doubtful cases and the presence of crystals on the outside of the cells makes judgment difficult.

The reversibility of the citrate effect was also studied. Leaves immersed in citrate for 10 minutes were restored to pond water or to 0.02M $CaCl_2$ for 10 minutes. They were then plasmolyzed in 0.05M sucrose and deplasmolyzed in distilled water. Crystals were obtained in these leaves; in greater abundance in the leaves that had been treated with the pure Ca salt solution. Tests with the electric current also showed that the effect of the citrate could be reversed by treatment with pond water or calcium solution.

The Effect of Isotonic Salt Solutions

Certain isotonic salt solutions are considered to be stimulating agents. Sodium salt solutions in particular cause muscles to twitch, and favor the action of other stimulants on *Arbacia* eggs. One of us (*cf.* Heilbrunn, Mazia, and Steinbach, 1934) has shown that in the latter case, isotonic NaCl caused an increase in the free Ca concentration of the cell interior.

Elodea leaves were washed and allowed to stand in 0.13M NaCl, 0.13M KCl, and 0.08M $CaCl_2$. They were examined at various times over 24 hours. The cells in NaCl stopped streaming first; at 18 hours the protoplasm was barely moving. At 18 hours, in the $CaCl_2$ solution, the protoplasm was streaming without carrying the chloroplasts, while in KCl the chloroplasts were in motion. No crystals were produced in any of these cells. The experiment was repeated a number of times; in no case was calcium oxalate precipitation observed.

Other Toxic Agents

Having found effective so many agents which, although stimulants, are also injurious, it is necessary to show that injurious agents which are not considered stimulants do not have the same effects as stimulants.

A number of such agents were tried. All of them caused the death of the cells without the production of crystals. They will simply be listed:

1. Ninety-five per cent ethyl alcohol.
2. Cold.
   - *a.* Zero degrees centigrade.
   - *b.* Temperature of solid CO_2.
3. Heat.
   - *a.* Seventy degrees centigrade.
   - *b.* One hundred degrees centigrade.
4. NH_4OH—0.01M.
5. $CuSO_4$—0.01M.

The Effect of Anesthetics

If the release of Ca ion is a general concomitant of the action of stimulants on cells, it is important to know whether anesthetics can prevent this release.

The anesthetics used were ethyl ether, chloral hydrate, and ethyl urethane. Their effect on the response to electrical stimulation and plasmolysis-deplasmolysis was investigated.

Leaves were placed in stoppered test tubes containing 1, 2, 3, 4, and 5 per cent solutions of ethyl ether in distilled water. They were removed after various times of immersion, and their reaction to currents of 1 milliampere was observed. In 1 per cent ether, even after 40 minutes immersion, the electric current caused the formation of crystals to the same extent as in the controls. The same result was obtained in 2 per cent and 3 per cent solutions. In 4 per cent ether the cells reacted like the controls for 20 minutes, then they appeared coagulated and no crystals were produced. Subsequent immersion in pond water for 30 minutes did not restore their responsiveness to the direct current.

More severe treatment, such as 30 minutes in 5 per cent ether, itself caused the production of small tetragonal crystals in most of the cells. The cells were dead; they could not be plasmolyzed and the amount of calcium oxalate could not be increased by applying electric currents.

The sequence of events could be more easily studied in the chloral hydrate experiments. Ten minutes in a 2 per cent solution did not prevent crystal formation. Longer treatment with this solution (13–14 minutes) caused the formation of large crystals, but passage of the electric current at this stage resulted in the formation of more crystals, concentrated at the anodal end. Still longer treatment with the anesthetic caused crystal formation accompanied by the death of the cells; subsequent treatment with the electric current produced no more crystals. A considerable number of experiments with chloral hydrate established the fact that severe treatments—use of solutions stronger than 2 per cent—cause the formation of large crystals, associated with the rapid death of the cell.

In the study of the effects of anesthetics on plasmolysis-deplasmolysis, the anesthetic was dissolved in the plasmolyzing agent. For example, cells were plasmolyzed in 0.2M NaCl containing 2 per cent ethyl urethane, and deplasmolyzed in pond water. Crystals were produced to the same extent as in the controls. The cells were exposed for as long as 30 minutes to the anesthetic, without effect.

The theoretical implications of these results will be considered in the general discussion.

DISCUSSION

Our results seem to us to uphold the hypothesis that when cells are stimulated, the free Ca concentration within the cell increases. We have shown the applicability of this hypothesis to the *Elodea* cell in its reaction to the most important stimulating agents. More complete verification of the hypothesis would depend on similar studies on widely different types of plant and animal cells.

However, the interpretation of the experimental production of calcium oxalate in plant cells can be examined more closely. The bulk of the free oxalate is in the vacuole; some may be in the protoplasm. Only a trace of Ca could be in a dissolved state in the vacuole unless the pH is 4 or less, at which reaction calcium oxalate is difficult to precipitate. Therefore, in our experiments, the Ca must come from the rest of the cell or from the surrounding medium. The latter possibility is eliminated by experimental results, discussed on p. 316.

Granted that the Ca comes from the protoplasm, it remains to be shown why calcium oxalate does not precipitate in the vacuole until the cell is subjected to the action of stimulating agents. There are two possibilities: (1) The vacuole membrane is impermeable to Ca, but becomes permeable at the moment of stimulation. (2) There is no considerable concentration of free Ca in the protoplasm, but bound Ca becomes free when stimulating agents are applied.

The first alternative faces two difficulties. In experiments in which the plasmalemma ruptures in Ca-containing solutions the vacuole immediately becomes packed with crystals. The vacuole membrane, therefore, seems to be very permeable to Ca, although this experiment does not prove certainly that this membrane is permeable when the plasmalemma is intact.

A second difficulty is that the cytoplasm seems to contain some free oxalate, which would bind any pre-existing free Ca. It could be shown experimentally that isolated tonoplasts are permeable to oxalate. If they are washed for an hour in distilled water, and Ca is added, no crystals are formed in the tonoplasts, although they can be formed before the washing. The observation recorded on p. 311, that on electrical disintegration of the plasmalemma in sugar solution some crystals are formed outside the vacuole, also supports the idea that the protoplasm contains oxalate. Finally, the oxalate must originally form in the cytoplasm; and would be precipitated as it formed if free Ca were present.

These facts support the second alternative, that the Ca which precipitates the oxalate in our experiments is released from a bound state on stimulation. Here the experiments with oxalate (and citrate) must be considered. The plasmalemma is relatively impermeable to sodium oxalate; cells plasmolyze in hypertonic solutions of this salt and do not deplasmolyze in a short time. Therefore, whenever cells are washed in oxalate, only the outer layer is affected. This is better proved by the fact that the effect of oxalate and citrate can be reversed by solutions containing Ca; if there is doubt about the relative impermeability of the cell to these ions, there is at least less doubt (considering the observations recorded on p. 318) about its relative impermeability to Ca.

If oxalate and citrate, in removing Ca from the outer layer of the cell, prevent the precipitation of calcium oxalate by agents which normally cause it, it follows that it is just this Ca in the outer surface that becomes free and forms the crystals in the vacuole. Oxalate and citrate can remove Ca from its combination with proteins. In ordinary analysis all the Ca in blood serum is precipitated by oxalate, although 50 per cent of it is bound. McLean and Hastings, cited above, have shown the same to be true for citrate.

In this discussion we may not have considered all the possibilities. The argument as it stands, however, indicates that when stimulants act on the *Elodea* cell, they act primarily on the non-diffusible calcium complexes in the cell exterior, releasing free Ca into the interior, some of which precipitates with the free oxalate that is present in the cell interior. This is the argument advanced by Heilbrunn and Daugherty (1933), as an interpretation of the viscosity changes in the exterior and interior protoplasm of *Amœba* under the influence of ultraviolet rays. Our direct results, therefore, support their contentions so far as this one reaction—Ca release—is concerned.

In considering our results in relation to the Heilbrunn theory, the results of the experiments with anesthetics must be explained. Fortunately, Daugherty (1936; in press) now presents evidence from viscosity measurements that is in accord with our evidence, and yet predictable from the Heilbrunn theory. She has found that the viscosity changes which she interprets on the basis of Ca changes are not affected by anesthetics. What is affected is the "clotting" reaction that normally results from the Ca release; Heilbrunn (1934) had already shown that this reaction was prevented by anesthetics in the presence of an adequate amount of free Ca.

Summary

We have attempted in a general way to test the hypothesis that a primary action of stimulants on cells is to cause an increase in the free Ca concentration within the cell.

1. The formation of crystals of calcium oxalate in the *Elodea* cell may be used as an intracellular method for detecting free Ca.
2. These crystals can be positively identified as calcium oxalate.
3. Condenser discharges and continuous direct currents cause the formation of crystals. The crystals are always formed at the anodal end of the cell.
4. Previous washing in solutions of oxalates and citrates prevents the formation of these crystals.
5. Ultraviolet irradiation causes the formation of calcium oxalate crystals. Previous washing in oxalate prevents this.
6. Mechanical stimulation causes the formation of crystals.
7. Heat treatment within the temperature range 40°–55° causes the formation of crystals. Oxalate prevents this.
8. Plasmolysis followed by deplasmolysis causes the formation of crystals. Oxalate and citrate prevent it.
9. The effect of the oxalate and citrate may be reversed by subsequent immersion in Ca-containing solutions.
10. Isotonic salt solutions, and a series of toxic agents, do not cause the formation of crystals.
11. Anesthetics in non-lethal doses do not prevent the formation of crystals by electric currents or osmotic changes.
12. The results are shown to be consistent with the idea that when stimulants act on these cells, they cause a release of Ca from combinations located in the periphery of the cells.

BIBLIOGRAPHY

Angerer, C. A., 1936. Effect of mechanical agitation on the relative viscosity of Amœba protoplasm. *Jour. Cell. and Comp. Physiol.* (in press).

BETHE, A., 1915. Kapillarchemische (kapillarelektrische) Vorgänge als Grundlage einer allgemeinen Erregungstheorie. *Pflüger's Arch.*, **163**: 147.

BLINKS, L. R., 1932. Migration of anthocyan pigment in plant cells during the flow of electric current, and reversal by acids and alkalies. *Proc. Soc. Exp. Biol. and Med.*, **29**: 1186.

CHAMOT, E. M., AND C. W. MASON, 1930. Handbook of Chemical Microscopy. Vol. I. John Wiley and Sons. New York.

DAUGHERTY, KATHRYN, 1936. The action of anesthetics on Amœba protoplasm. (In press.)

HEILBRUNN, L. V., 1928. The Colloid Chemistry of Protoplasm. G. Borntraeger. Berlin.

HEILBRUNN, L. V., 1934. The effect of anesthetics on the surface precipitation reaction. *Biol. Bull.*, **66**: 264.

HEILBRUNN, L. V., AND KATHRYN DAUGHERTY, 1933. The action of ultraviolet rays on Amœba protoplasm. *Protoplasma*, **18**: 596.

HEILBRUNN, L. V., D. MAZIA, AND H. B. STEINBACH, 1934. Free and bound calcium in stimulation and injury. *Anat. Rec.*, **60**: (suppl.) 32.

HEILBRUNN, L. V., AND R. A. YOUNG, 1930. The action of ultraviolet rays on Arbacia egg protoplasm. *Physiol. Zoöl.*, **3**: 330.

KÜHNE, W., 1864. Untersuchungen ueber das Protoplasma und die Contractilität. Leipzig.

KÜSTER, E., 1929. Pathologie der Pflanzenzelle. Teil I. Pathologie des Protoplasmas. G. Borntraeger. Berlin.

LEE, A. B., 1900. The Microtomist's Vade-mecum. P. Blakiston Sons and Co. Fifth Edition. Philadelphia.

LEPESCHKIN, W. W., 1936. Fortschritte der Kolloidchemie des Protoplasmas in den letzten zehn Jahren. III. *Protoplasm*, **25**: 301.

McLEAN, F. C., AND A. B. HASTINGS, 1935. The state of calcium in the fluids of the body. I. The conditions affecting the ionization of calcium. *Jour. Biol. Chem.*, **108**: 285.

MALPIGHI, M., 1675 and 1679. Die Anatomie der Pflanzen. (Ostwald's Klassiker, No. 120. Emgelmann, Leipzig.)

MAST, S. O., 1931. The nature of the action of electricity in producing response and injury in Amœba proteus (Leidy) and the effect of electricity on the viscosity of protoplasm. *Zeitschr. f. vergl. Physiol.*, **15**: 309.

NADSON, G. A., ET E. ROCHLINE-GLEICHGEWICHT, 1927. De la Formation des Cristaux de l'Oxalate de Calcium dans les Cellules Végétales sous l'Influence des Rayons Ultraviolettes (in Russian). *Vestnik Rentgenologii i Radiologii*, **5**: 415.

——, 1928. Apparition des Cristaux d'Oxalate de Calcium dans les Cellules Végétales sous l'Influence de la Radiation Ultra-violette. *Compt. Rend. Soc. Biol.*, **98**: 363.

OSTERHOUT, W. J. V., 1910. On the penetration of inorganic salts into living protoplasm. *Zeitschr. f. Physik. Chem.*, **70**: 408.

PATSCHOVSKY, N., 1920. Studien über Nachweis und Lokalisierung, Verbreitung und Bedeutung der Oxalsäure im Pflanzenorganismus. *Beih. z. Bot. Zentralbl.*, **37** (Abt. 1): 259.

ROCHLINE-GLEICHGEWICHT, E. J., 1930. L'Influence de l'Emanation de Radium sur la Cellule Chlorophyllifere. (In Russian.) *Vestnik Rentgenologii i Radiologii*, **8**: 387.

RUBINSTEIN, D. L., UND VERA USPENSKAJA, 1934. Über den isoelektrischen Punkt der pflanzlichen Plasmahaut. *Protoplasma*, **21**: 191.

WEIS, A., 1925. Beiträge zur Kenntnis der Plasmahaut. *Planta*, **1**: 145.

WHERRY, E. T., AND G. L. KEENAN, 1923. Occurrence and forms of calcium oxalate crystals in official crude drugs. *Jour. Am. Pharmaceut. Assoc.*, **12**: 301.

OBSERVATIONS ON THE MULTIPLICATION OF BACTERIA IN DIFFERENT VOLUMES OF STORED SEA WATER AND THE INFLUENCE OF OXYGEN TENSION AND SOLID SURFACES

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INTRODUCTION

The storage of raw sea water in the laboratory is usually accompanied by increased bacterial activity. This may be manifested by the evolution of carbon dioxide, increase in the hydrogen-ion concentration, oxygen consumption, ammonia production, nitrate reduction, or by bacterial multiplication. Waksman and Carey (1935*a*) report that the bacterial population of raw sea water increases from a few hundred bacteria per cc. to millions per cc. following a few days storage in the laboratory. They (1935*b*) attribute this great increase primarily to the dying out of Protozoa and other marine animals which devour bacteria and to the modification of "certain controlling factors injurious to free bacterial development." Keys, Christensen, and Krogh (1935), who observed similar changes, believe that the activity of bacteria in the sea is limited by the "extreme stability of the ocean as a chemical and physical factor."

In their preliminary studies on sampling methods, ZoBell and Feltham (1934) observed a great increase in the number of bacteria in samples of stored sea water even at refrigeration temperatures and also a diminution in the number of varieties. These changes were found to occur less rapidly in large samples than in small ones. The effect of volume on the multiplication of bacteria in stored water was recognized by both Harvey (1925) and Föyn and Gran (1928), who mention the work of Whipple on stored fresh water. Whipple (1901) noted that the smaller the volume of fresh water, the greater is the rate of bacterial multiplication in it. After 24 hours of storage he found 300 bacteria per cc. in a gallon, 7,020 per cc. in a pint, and 41,400 per cc. in 2 ounces of a representative water sample having an initial count of 77 bacteria per cc.

While it is impracticable either to collect or to store samples of sea water which are large enough to prevent for more than a few hours undesirable changes in its bacterial and chemical composition, the fact that the volume seems to influence the activity of bacteria may be of significance. For example, in one of their experiments on its organic-matter content Keys, Christensen, and Krogh (1935) ultrafiltered small amounts of sea water free of all particulate and colloidal organic matter. After its inoculation with normal flora there was just as much bacterial multiplication in it as in ordinary filtered sea water. Is it possible that the results were influenced by the smallness of the volume of ultra-filtered sea water? Other questions arise: Is the great increase in the bacterial population which accompanies the storage of sea water in any way related to its confinement to a small volume, and if so, to what extent and how? Also, if bacterial activity is influenced by volume, to what extent do controlled test-tube experiments indicate what is actually occurring in the ocean? In summarizing the factors which influence bacterial growth curves Rahn (1932) concludes that the volume of the media has no influence on the multiplication of bacteria. Woodruff (1911) and Greenleaf (1926) report that certain Protozoa multiply faster in large volumes than in small ones while Robertson (1922) found the antithesis of this.

Experimental Methods

Sea water was collected in glass battery jars from the end of the Scripps Institution pier which extends 1,000 feet seaward where the mean depth of the water is about 20 feet. Despite its proximity to land, the water is chemically and biologically quite typical of marine conditions because of the oceanic circulation and the freedom from terrestrial drainage. The water was siphoned into a Buchner 4G sinter-glass filter and filtered by gravity into a 10-gallon bottle. The filtration was merely to remove particulate matter and to permit the oxygen tension to come to equilibrium with the atmosphere. After thoroughly mixing the water by vigorous shaking to insure greater uniformity in its composition, it was siphoned into different sizes of Pyrex bottles or flasks. Some were filled to capacity and glass-stoppered and others were only partly filled, thus leaving the water in contact with air. Chemically clean, sterilized apparatus and aseptic technique were used throughout the experiments.

The bacterial population of the water was determined by plating procedures immediately after its collection from the sea, again after it had been distributed to bottles of different sizes and at periodic intervals thereafter. Two or more dilutions of each sample were plated in duplicate. Sterile sea water was used as the diluent. The following nutrient agar has proved to yield larger plate counts than any of several other formulæ which have been tried:

Proteose peptone	2.0 grams
Bacto peptone	3.0 grams
Beef extract	2.0 grams
$FeCl_3 \cdot 6H_2O$	0.2 gram
Bacto agar	12.0 grams
Sea water	1,000.0 cc.

After dissolving the agar by steaming, the reaction was adjusted to pH 7.8 with 1/N NaOH and the medium was clarified by filtering it through glass wool. The oxygen content of the sea water was estimated with Winkler's reagents.

RESULTS

In the first series of experiments glass-stoppered Pyrex bottles having similar shapes, proportional dimensions and different capacities were filled about five-sixths full with 10, 100, 1,000, and 10,000 cc. of raw sea water. The air in the upper one-sixth of the bottles provided for the saturation of the water with oxygen and it permitted more thorough mixing when the water was shaken at the time of sampling. The bottled water was stored in a dark constant temperature room at 16° C. Samples were withdrawn daily for plate counts. Fig. 1 shows graphically

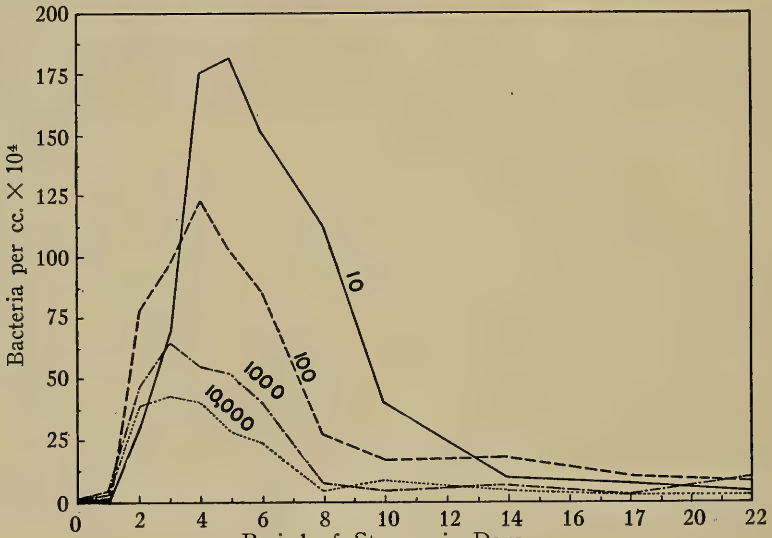

FIG. 1. Influence of volume on the multiplication of bacteria in stored sea water. Growth curve in 10 cc. represented by solid line, 100 cc. by dashed line, 1,000 cc. by dot-dashed line and 10,000 cc. by dotted line.

the number of bacteria which were found per cc. of water after different periods of storage in bottles of different sizes.

This experiment has been repeated several times under a variety of conditions. Almost invariably the densest bacterial populations appear in the smallest volumes of sea water. Table I summarizes the maximum plate counts found in four different volumes of three different samples of sea water. It is impossible to duplicate results in the usual sense of the term because each different sample of sea water varies in chemical and biological composition. The initial plate counts of the water collected for these experiments during the last four years has varied from less than 100 bacteria per cc. to nearly 30,000 per cc. Of greater importance is the organic-matter content of sea water which fluctuates in quality and quantity (Bond, 1933). Waksman and Carey (1935*b*) have shown that the amount and specific nature of organic substances control the abundance of the bacterial population developing in sea water.

Table I

Maximum number of bacteria found per cc. of sea water after storage at 16° C. in glass-stoppered Pyrex bottles of different sizes.

Sample No.	86	149	165
Date collected	9/14/35	1/9/36	2/6/36
Initial count per cc.	930	470	2,570
Volume of sea water	Plate count per cc. following storage		
10 cc.	1,050,000	1,960,000	1,810,000
100 cc.	680,000	1,240,000	1,070,000
1,000 cc.	251,000	645,000	615,000
10,000 cc.	164,000	255,000	340,000

As indicated by Fig. 1, during the *maximum stationary phase* of the growth curves (Winslow, 1928) there may be twice as many bacteria per cc. in small volumes as in volumes ten times as large. It requires from three to six days storage at 16° C. for the maximum to be attained. The densest bacterial populations which appear in the smallest volumes of stored water require the longest periods of incubation. As a general rule, the growth curves in the smaller volumes are still in the phase of *logarithmic increase* while the bacterial populations in the larger volumes are declining. Although there is evidence of multiplication within eight hours after the water is bottled, little or no difference is found in the density of the bacterial populations in the different volumes during the first two days. During the *phase of decrease* the bacterial populations in the smaller volumes continue to exceed those in the larger volumes until the *phase of readjustment* is reached 8 to 18 days after the sea water was first stored at 16° C. During the latter phase the effect of volume on the plate counts disappears entirely as the bacterial populations fluctuate irregularly from a few thousand to over a hundred thousand bacteria per cc. At least a few thousand bacteria per cc. can be demonstrated in any volume of stored sea water for several weeks. After four years storage at 2 to 6° C. in a 100-cc. glass-stoppered bottle there were 209,000 bacteria per cc. of sea water.

In some experiments 1.0 cc. of sea water was stored in test tubes. Each successive day two of these were analyzed by washing them out several times with a 99 cc. dilution water blank. The wash water was appropriately diluted and plated. Also 1.0 cc. volumes were stored in 1.0 cc. pipettes which were filled to the mark, and the water retained by forcing the end of the pipette into a rubber stopper having a small hole bored halfway through it. These were similarly analyzed in duplicate by delivering the contents into 99-cc. water blanks and then washing the pipettes by filling and emptying several times. Table II shows the average number of bacteria found under both conditions of storage on each day, and for comparison the corresponding plate counts in 10 cc. and 100 cc. volumes of identical sea water are also given. It will be observed that again the smallest volume of sea water supported the largest bacterial populations. Following the storage of some 1.0 cc. samples of sea water, more than 4,000,000 bacteria have been demonstrated by plate counts whereas the maximum bacterial populations in 10-liter samples of the water have been only 2 to 5 per cent as much. The same sea water stored in the interstitial spaces of glass beads or sand which may be regarded as multiple minute volumes supports bacterial populations exceeding ten million per cc.

TABLE II

Number of bacteria per cc. of sea water following storage at 16° C. of different volumes in various receptacles.

Storage period	1.0 cc. in pipettes	1.0 cc. in test tubes	10 cc. in test tubes	100 cc. in bottles
Start	280	280	280	280
2 days	131,000	128,000	115,000	275,000
3 days	1,210,000	1,340,000	570,000	580,000
4 days	1,230,000	1,410,000	955,000	640,000
5 days	1,510,000	1,895,000	640,000	420,000
6 days	1,300,000	1,510,000	280,000	250,000

Inasmuch as it is quite obvious that in small volumes of water the temperature would change more rapidly than in large volumes, precautions were taken to maintain the different volumes of water at the same temperature. First, all of the water in the 10-gallon reservoir was brought to the desired temperature. The change was usually slight because the temperature of the sea water which was collected for most of the experiments was 13 to 16° and it was stored in a constant temperature room at 16°. In order to avoid thermic disturbances, in some experiments the sea water was stored in a water bath at exactly the same temperature as when collected. Later, receptacles of different capacities were filled with filtered sea water on the end of the pier and these were suspended in the sea, 4 to 5 meters below the surface. The bottles were hauled upon the pier each day to permit sampling. Again many more bacteria per cc. were found in the small bottles than in the larger bottles just as was found in the laboratory. Finally, bottles full of sea water were submerged in the sea stoppered with a diatomaceous earth filter candle in a rubber stopper. In other bottles the mouth was narrowed by stoppering with a one-hole rubber stopper in which was inserted a 5 mm. bore glass tube bent at a sharp angle. Under both conditions the bacterial populations in 100 cc. bottles increased to nearly a million per cc. and in the 10-liter carboy to over a hundred thousand bacteria per cc., whereas the sea water *in situ* in which the bottles were submerged contained less than a thousand bacteria per cc.

When sea water is analyzed immediately after collection, from 25 to 35 different species of bacteria can be recognized by a careful scrutinization of their colonial characteristics. Actually 96 different species of marine bacteria have been isolated and culturally, morphologically and physiologically characterized. Almost before there is evidence of multiplication in stored sea water, the number of species begins to decrease until at the time the *maximum stationary phase* in the growth curve is reached, only nine or ten different species are detectable on the plates. Quantitative data cannot be given, but it is our impression that there are usually fewer predominating species in the smaller volumes than in the larger volumes of stored sea water. That many species disappear during storage and do not merely escape detection by being outnumbered by others which multiply rapidly is indicated by the fact that during the *readjustment phase* when the populations have decreased to a few thousand bacteria per cc., only four or five species can be recognized on the plates regardless of the volume.

Effect of Oxygen

Rahn (1932) states that with the same organism in the same medium the final amount of growth " will be proportional to the volume of the medium unless the volume has had an influence upon the rate of oxygen penetration into the medium." Whipple (1901) attributed the more rapid multiplication of bacteria in small samples of fresh water to the greater oxygen content of the water. However, in his experiments Whipple added 0.0005 per cent peptone to the water samples to hasten bacterial multiplication, and the additional peptone would increase the oxygen consumption. Likewise Rahn's conclusions are based upon results obtained in media rich in organic matter in which there would be appreciable oxygen consumption. There is very little organic matter in sea water. According to Krogh (1931) it contains less than 10 mgm. of organic matter per liter, much of which cannot be utilized by bacteria. Waksman and Carey (1935*a*) as well as Keys, Christensen, and Krogh (1935) find that bacterial activity is arrested by the depletion of available organic matter in stored sea water before half of the dissolved oxygen is consumed.

TABLE III

The initial and maximum plate counts per cc. of sea water stored in partly filled bottles of different sizes and the oxygen content of similar water in open bottles after 7 days quiescent storage at 16° C.

Volume of sea water	Plate count per cc.		Oxygen in cc. per liter	
	Initial	Maximum	Initial	After 7 days
cc.			*cc.*	*cc.*
100	460	1,360,000	5.46	5.14
1,000	460	670,000	5.46	5.39
10,000	460	485,000	5.46	5.38

Those who have criticized our paradoxical observations that small volumes of stored sea water support larger bacterial populations than large volumes suggest that the explanation will most likely be found in the difference in the oxygen content of the water. Therefore this factor has been carefully considered from various angles.

In bottles which were only partly filled with sea water it was found that the contact with the air plus the daily shaking at the time the samples were taken kept the water virtually saturated with oxygen. The oxygen content of the water under these conditions was decreased only slightly even when not shaken. This is illustrated by the data in Table III, which give the maximum bacterial populations which were attained during the storage of different volumes of sea water and the oxygen content of similar water after 7 days quiescent storage.

In the next series of experiments the bottles were filled full, glass-stoppered and submerged in a water bath at 16° C. Duplicate 100 cc. and one-liter bottles (actually somewhat more than 120 and 1,200 cc.

respectively) were analyzed each day for their bacterial and oxygen content. Samples were withdrawn from the 10,000 cc. bottle each day by means of a series of siphons and traps which permitted the withdrawn samples to be replaced by similar sea water from another receptacle without coming into contact with air. The pertinent data of a representative experiment are summarized in Table IV. In every case the oxygen consumption per unit of water was greatest in the smallest volumes in which were found the largest bacterial populations. This would seem to indicate that the oxygen content of the water does not account for the increased bacterial activity in small volumes of sea water.

It may be of interest to note that oxygen consumption in any volume of stored sea water parallels the growth curves until the *maximum stationary phase* is reached. However, during the period of *logarithmic decrease,* oxygen consumption is not retarded but continues in a straight line at the same rate as it does during the *logarithmic phase* of growth. This is illustrated by Fig. 2 which gives the growth and oxygen-consumption curves for 100 and 1,000 cc. of stored sea water. Although there were approximately the same number of viable bacteria per cc. in both volumes as demonstrated by plate counts after the tenth day, twice as much oxygen was consumed per unit of water in the 100-cc. receptacle as in the 1,000-cc. receptacle. This may indicate that there are many more bacteria which are respiring than the number which can be demonstrated by plating procedures.

TABLE IV

The maximum plate counts per cc. of sea water stored in completely filled glass-stoppered bottles of different capacities and the oxygen content of the water after 18 days storage at 16° C.

Volume of sea water	Plate count per cc.		Oxygen per liter		Oxygen consumed per liter
	Initial	Maximum	Initial	18 days	
cc.			*cc.*	*cc.*	*cc.*
100	276	1,010,000	5.40	2.87	2.53
1,000	276	580,000	5.40	4.09	1.31
10,000	276	370,000	5.40	4.43	0.97

The oxygen-consumption curves do not continue as straight lines until all the oxygen is depleted, as may be inferred from Fig. 2. Instead the curves start to flatten out between the fifteenth and twenty-first days, and oxygen is consumed only very slowly after 28 days storage at 16° C. Oxygen consumption in liter samples continues at a slower rate and for longer periods of time than in 100-cc. samples.

Oxygen consumption in 10-liter samples has not been followed for more than three weeks. Regardless of the volume of the samples, oxygen is not depleted from sea water which is initially saturated (5.0 to 5.8 cc. oxygen per liter) unless utilizable organic matter is added. Respiration as indicated by oxygen consumption virtually ceases while there is still between 2 and 3 cc. of oxygen per liter in solution. However, upon the addition of only small amounts of organic matter, the oxygen content is rapidly reduced to zero in any volume of sea water from which atmospheric oxygen is excluded. Strips of filter paper and even agar-agar suffice. It has been shown by ZoBell and Anderson (1936) that bacteria not only deplete the oxygen from marine bottom deposits but that they actually create an oxygen deficit. As will be discussed below, the volume effects disappear when more than a few milligrams of utilizable organic matter per liter is added to the sea water.

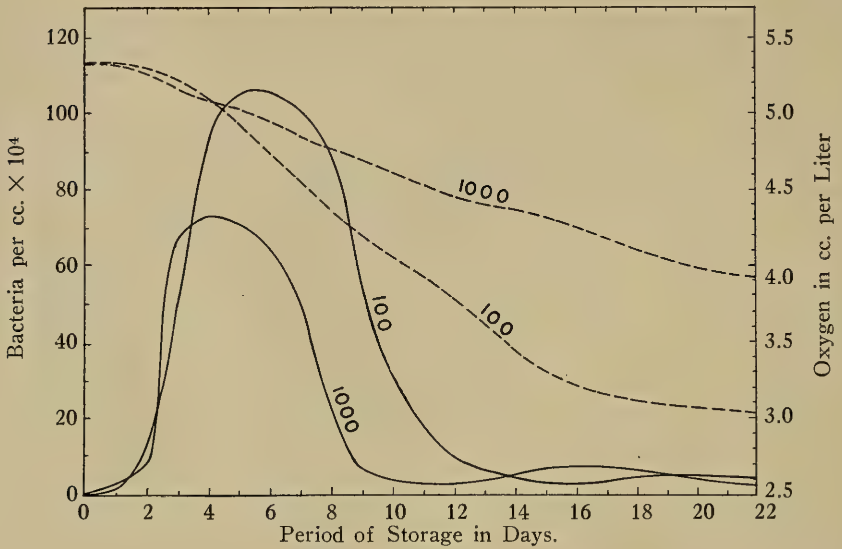

FIG. 2. Influence of volume on oxygen consumption in stored sea water. Solid lines represent growth curves and dashed lines oxygen consumption in 100 and 1,000 cc. respectively.

As further evidence that the differential penetration of oxygen in sea water in receptacles of different capacities does not account for the increased bacterial activity in small volumes, sea water having different initial oxygen contents was stored. Twenty liters of freshly collected sea water was deoxygenated by holding it for two hours at a pressure reduced to less than 1 cm. of mercury with a High-Vac pump during which time the water was agitated. Then pure nitrogen was used to force the water into 120-cc. bottles which were immediately stoppered and sealed. Under these conditions sea water containing only 0.59 cc. oxygen per liter was obtained. Some of this deoxygenated water was mixed with oxygen-saturated sea water to give a series having an initial oxygen content of 3.08 cc. per liter. As a control some of the deoxygenated water was saturated with oxygen by shaking it with air. It then contained 5.42 cc. of oxygen per liter. The tightly stoppered bottles were submerged in a water bath at 16° C. After different periods of incubation duplicate bottles were analyzed for bacteria and oxygen content. Table V presents the findings.

There was considerable bacterial activity in the deoxygenated water which contained only 0.59 cc. oxygen per liter as manifested by the rapid increase in the bacterial population and the depletion of the oxygen. As a matter of fact, the multiplication rate in the deoxygenated water was only slightly less than in the oxygen-saturated water until the oxygen was depleted. Likewise it will be observed that the amount of oxygen consumed during the first seven days was virtually the same regardless of the initial oxygen content of the water. Bacterial multiplication in sea water having a low oxygen tension was expected because most marine bacteria are microærophiles and many of them are facultative anaerobes. However, the rate of multiplication and oxygen-consumption in the water of low oxygen tension proved to be surprisingly rapid and these results are reported only after having been repeated.

TABLE V

Bacterial and oxygen content of sea water initially containing different concentrations of dissolved oxygen after storage at 16° C. in 120-cc. bottles.

Storage period	Oxygen-saturated water		Partly saturated water		Deoxygenated water	
	Bacteria per cc.	Oxygen in cc. per liter	Bacteria per cc.	Oxygen in cc. per liter	Bacteria per cc.	Oxygen in cc. per liter
Start	1,190	5.42	1,230	3.08	1,240	0.59
3 days	264,000	5.12	224,000	2.91	216,000	0.42
5 days	1,458,000	4.68	1,170,000	2.48	660,000	0.22
7 days	792,000	4.35	1,226,000	2.26	683,000	0.00
10 days	116,000	3.82	469,000	1.65	152,000	0.00
18 days	77,000	2.90	17,000	1.19	28,000	0.00

Finally, similar volumes of water were placed in receptacles of different shapes so that different surface areas were exposed to the air. For example, 500 cc. quantities of water were placed in liter Roux flasks, in 500 cc. graduate cylinders and in glass tubes 2.1 cm. in diameter by

140 cm. long. When a Roux flask was placed on its side, 220 sq. cm. of water surface was exposed to air. An identical volume of water in a Roux flask stood on end exposed only 40 sq. cm. of water surface to air. In the 500-cc. cylinder 16 sq. cm. was exposed to air and in the 2.1 cm. tube only 3.2 sq. cm. of the water surface was exposed to air. Although it is recognized that sampling difficulties introduced certain errors, the bacterial populations were found to be in no way related to the area of water surface exposed to air. However, a relationship was noted between the area of glass surface exposed to water and the bacterial populations in stored sea water.

EFFECT OF SOLID SURFACE

It is well known that the smaller the capacity of similarly shaped receptacles the greater is the surface area per unit volume. This may be illustrated by the data in Table VI which gives the dimensions of the receptacles which have been in use in the foregoing experiments. The average maximum number of bacteria which have been found in these receptacles in three different experiments is also given. It will be observed that the densest bacterial populations appear in the bottles of water which offer the largest area of glass surface per unit volume of water. As reported above, this was likewise found to be true when the volume was kept constant (500 cc.) and the shape of the receptacles varied to give different glass surface areas.

TABLE VI

*Volume of sea water stored in different receptacles, area of solid surface in contact with the water, ratio of volume in cc. to area in sq. cm. and the average * maximum bacterial populations per cc. after storage at 16° C.*

Volume of sea water in cc.	Solid surface in sq. cm.	Ratio of cc. : sq. cm.	Average maximum * bacteria per cc.
14............	37	1 : 2.64	1,863,000
120............	148	1 : 1.23	1,070,000
1,225............	640	1 : 0.52	553,000
13,220............	3174	1 : 0.24	261,000

* Average of three different experiments.

Next receptacles were selected so that their volumes varied greatly but the ratios of glass surface to volume remained practically constant. This was accomplished by using different lengths of glass tubing 2.1 cm. in diameter, one end of which was sealed and the other end stoppered. From 28 to 925 cc. of sea water was stored in such cylinders and within the limits of experimental error the growth curves ran practically parallel. Table VII summarizes typical bacteriological findings in experiments in which receptacles of assorted capacities and shapes giving a wide range of ratios of volume to solid surface were used for storing sea water. Prior to being used all the glassware was aged in sea water, cleaned with hot sulfuric acid dichromate solution, rinsed with distilled water and sterilized. While the expression of the relationship of glass surface to bacterial populations defies exact mathematical expression, it will be noted that: in general, the larger the ratio of volume to surface area, the larger the bacterial populations regardless of the total volume of stored sea water.

TABLE VII

Volume of sea water stored in different receptacles, area of solid surface in contact with the water, ratio of volume in cc. to area in sq. cm. and the average maximum bacterial population following storage at 16° C.

Receptacle	Volume of sea water	Area of solid surface	Ratio of cc. : sq. cm.	Bacteria per cc.
1.0 cc. pipette	1.0 cc.	9.9 sq. cm.	1 : 9.9	2,480,000
Blake bottle on side	10.0 cc.	98.7 sq. cm.	1 : 9.9	3,120,000
1.8 cm. test tube	1.0 cc.	4.1 sq. cm.	1 : 4.1	1,895,000
2.5 cm. test tube	10.0 cc.	20.3 sq. cm.	1 : 2.0	855,000
2.1 cm. glass tube, 8 cm. long	28.0 cc.	50.4 sq. cm.	1 : 1.8	970,000
2.1 cm. glass tube, 140 cm. long	550.0 cc.	925.0 sq. cm.	1 : 1.7	720,000
Nest of glass tubes, 140 cm. long *	410.0 cc.	2,556.0 sq. cm.	1 : 6.2	1,470,000
Liter bottle	1,225.0 cc.	640.0 sq. cm.	1 : 0.5	490,000
Liter bottle with 4,100 3 mm. glass beads	1,067.0 cc.	1,690.0 sq. cm.	1 : 1.7	642,000
Liter bottle with 1,350 cm. 3 mm. glass rods	1,099.0 cc.	1,840.0 sq. cm.	1 : 1.7	598,000

* The nest of glass tubes consisted of a piece of 2.1 cm. glass tubing 140 cm. long in which was placed three glass cylinders nearly as long and 1.6, 1.0 and 0.6 cm. in diameter respectively.

In the liter bottles in which the ratios of volume to solid surface were increased by placing a shallow layer of 3-mm. glass beads or glass rods in them, the plate counts were higher than in ordinary liter bottles but the increases were not proportional to the additional surface area nor were they as high as in other receptacles having the same ratios (see Table VII). This is considered as evidence that another factor besides solid surface is involved which influences the activity of bacteria in stored sea water. Further proof was obtained by covering the bottoms of liter bottles with enough glass wool or fine silica sand to give ratios exceeding 1 : 1,000. Following the storage of sea water under these conditions, the plate counts were only slightly higher than in ordinary liter bottles having ratios of only 1:0.5 and about the same as in liter bottles containing enough 3-mm. glass beads to give ratios of 1:1.7. The explanation is believed to be found in the proximity of the water to a solid surface or in the distribution of the solid surface throughout the water. For example, in a liter bottle whose diameter is 10.6 cm., part of the water is at least 5 cm. away from the nearest solid surface whereas in a 2.1 cm. tube, the greatest distance is only about 1 cm. and within the interstitial water between 3-mm. glass beads the greatest distance of any particles of water to the nearest solid surface is only a fraction of a millimeter. Thus while a layer of glass beads or sand in the bottom of the bottle increases the surface per unit volume, the additional solid surface affects only that part of the water which is in the proximity of the glass beads or sand.

Sea water stored within the interstices of small inert particles such as sand or glass beads supported larger bacterial populations than it did under any other condition of storage. Fine silica sand was treated with hot sulfuric acid dichromate cleaning solution and washed with distilled water until the reaction was neutral. The clean sand was then dried in an oven after which 25-gram portions were weighed into 100-cc. Pyrex bottles. These bottles containing sand were heated to 240° C. for 6 hours. When cool, the sand was moistened with 10 cc. of sea water which was just enough to barely cover the sand. After varying periods of storage the sea water and sand mixture was washed into a 900-cc. water blank with 90 cc. of sterile sea water. Plating dilutions of this revealed the presence of from six to twelve million bacteria per cc., whereas less than a million per cc. appeared in identical sea water when 10 cc. was stored in similar bottles without sand. Between four and six million bacteria per cc. were demonstrated in the sea water with which the small glass beads in a 100-cc. bottle were covered. Controls consisting of clean sand or glass beads covered with distilled water and inoculated with a mixed microflora showed no appreciable bacterial multiplication, thereby indicating that neither the sand nor the beads supplied utilizable nutrients. The high counts which are obtained when sea water is stored in sand or glass beads are attributed to the proximity of the water to solid surfaces. There may be even more bacterial multiplication under these conditions than is indicated by the plate counts because it is improbable that all of the viable bacteria are recovered from the sand or beads.

Early in this work it was recognized that marine bacteria have a tendency to adhere to solid surfaces. Consequently in all of the experiments the water was vigorously shaken prior to sampling in order to recover as many bacteria as possible. Usually the bottles were only partly filled with water to permit thorough mixing except in the experiments dealing with oxygen consumption in stored sea water. The 1- to 10-cc. volumes of stored sea water were poured directly into the dilution water and the receptacle in which the water had been stored was rinsed out several times. Receptacles which were filled to capacity were partly emptied into sterile vessels to permit shaking. The sea water stored in long glass tubes was poured out and in several times to wash the bacteria from the glass surfaces to which they adhere.

In order to show quantitatively the tendency of bacteria to adhere to the glass, 100 cc. of sea water was stored in 120-cc. bottles, two of which were analyzed daily. First, all of the water was transferred to another vessel without any shaking and this was appropriately diluted and plated.

Table VIII

Number of bacteria per cc. in 100 cc. of sea water poured from bottles in which it had been stored without shaking and the number per cc. in the first and second 100 cc. of sea water with which the original bottle was rinsed.

Storage period	Number of bacteria per cc.		
	Original water	First rinse water	Second rinse water
Start	2,800	0	0
2 days	65,000	48,000	2,600
3 days	108,000	67,000	3,900
4 days	220,000	90,000	10,600
5 days	278,000	122,000	6,450
6 days	270,000	136,000	10,200
10 days	10,000	120,000	9,600

Then the original bottle in which the sea water had been stored was thoroughly rinsed with 100 cc. of sterile sea water and this was poured off and plated. The rinsing process was repeated with a second 100 cc. of sterile water which was plated. The resulting plate counts are given in Table VIII. The data show that on an average nearly half of the bacteria in the bottles of stored sea water were stuck firmly enough to the walls of the bottle to resist being detached by pouring off the water without shaking.

The foregoing procedure fails to demonstrate all of the bacteria which are attached to the glass because many are not washed off by any amount of rinsing in water. After being rinsed with several 100-cc. portions of water, 20 cc. of melted nutrient agar cooled to 45° C. was poured into the original bottle in which the sea water had been stored and the agar was permitted to solidify on the inside periphery of the bottle Esmarch-tube fashion. Following incubation too many colonies developed to permit their enumeration.

Direct microscopic examination (Henrici, 1933) of slides which were suspended in liter bottles of stored sea water for 24 hours revealed the presence of over 100,000 bacteria per sq. cm. If bacteria were equally abundant on all of the glass surface within the bottle, it is calculated that for each cc. of water there was the equivalent of 84,000 bacteria attached to the glass while the water itself contained only 31,000 bacteria per cc. including those which could be dislodged by shaking vigorously the bottle of water. The proponderance of attached bacteria increases progressively during storage until they become too thick to enumerate with precision. It is recognized that all of the bacteria observed microscopically may not be alive and also that plate counts do not demonstrate all of the viable bacteria in the water. Therefore the data obtained by the two procedures are not directly comparable. However, the data conclusively demonstrate that large numbers of bacteria in stored sea water attach to glass surfaces. The development of micro-colonies on the slides immersed in sea water indicates that many of the attached bacteria are alive and multiplying. The respiration of these attached bacteria is believed to account for the oxygen consumption which continues to occur in stored sea water after the bacterial population of the water has dropped to a low level (see Fig. 2).

In these experiments the bacteria were not fixed to the slides by heat or otherwise. The criterion of periphytic bacteria is their ability to attach themselves so tenaciously to the slide that they are not dislodged by washing in water and the other staining procedures. According to ZoBell and Allen (1933) appreciable numbers of bacteria attach themselves to glass slides within 2 to 4 hours after being submerged in sea water. They (1935) found as many as 720,000 bacteria per sq. cm. after the slides had been suspended in the sea for 24 hours. Nearly a billion bacteria per sq. cm. have been observed on glass slides submerged in the sea for a week.

Discussion

The maximum bacterial population which sea water filtered free of particulate matter can support is probably between ten and one hundred million per cc. (including estimated periphytes). Bacterial multiplication in stored sea water is undoubtedly limited by organic matter because over a billion bacteria per cc. have been found in sea water enriched with 100 mgm. of peptone per liter. However, when enriched there is no apparent difference in the activity of bacteria in different volumes of sea water. The fact that the volume effects occur only in very dilute nutrient solutions may explain why exhaustive growth curve studies of other investigators have not shown volume of the media or solid surfaces to be a factor which influences bacterial multiplication. Most growth curve studies on saprophytes have been conducted in media containing at least 1,000 mgm. of organic matter per liter (Rahn, 1932). Using different carbon compounds Stephenson (1930) shows that 10 to 100 mgm. per liter is the minimum concentration in which *Bact. coli* and certain other bacteria can grow. It will be recalled that sea water contains less than 10 mgm. of organic matter per liter.

Multiplication and oxygen consumption are not the only bacterial activities in stored sea water which are influenced by volume or surface. It was found that the minimum concentration of organic matter which limits denitrification was considerably less in 10-cc. quantities of sea water than in 500-cc. quantities. This was not understood until the effect of solid surface on bacterial activities in dilute nutrient solutions was studied. Several Erlenmeyer flasks varying in size from 25 to 6,000 cc. were filled with raw sea water enriched with 10 mgm. peptone and 1.0 mgm. KNO_2 per liter. It was found that nitrite was depleted from the smallest flasks first and from the largest flasks last or not at all. When attempts were made to accelerate the reaction by adding 100 mgm. or more of peptone per liter, there was no difference between small and large receptacles in the rate of nitrite reduction. Changes in the pH and ammonia content likewise occur more rapidly in sea water stored in small receptacles than in identical sea water stored in large receptacles.

The volume effects are attributed to the favorable influence of solid surfaces and to the proximity of these surfaces to the water particles. Solid surfaces are believed to favor bacterial activity in dilute nutrient solutions in several ways. First, they provide a resting place for periphytes. In discussing the advantages of semi-solid media, Hitchens (1921) suggests that bacteria may need objects upon which to cling while growing. It is difficult to estimate what proportion of marine bacteria are periphytes but ZoBell and Allen (1933) have demonstrated that many species have periphytic tendencies and that at least some of them are obligate periphytes. This is in agreement with field observations. Lloyd (1930) calls attention to the fact that the saprophytic bacteria of the sea are not planktonic but are attached to suspended particles. Waksman and co-workers (1933) find a definite parallelism between the bacteria and plankton in sea water and they conclude that "bacteria exist only to a very limited extent in the free water of the sea, but are largely attached to the plankton organisms." This conclusion is corroborated by the literature cited by Benecke (1933) dealing with the distribution of bacteria in the sea.

Secondly, solid surfaces favor bacterial activity in dilute nutrient solutions because the nutrients may become concentrated in a film upon the solid surface due to adsorption or other kinds of physical attraction. Studies on the micro-organic films which form on glass slides soon after being submerged in sea water give some evidence of such a concentration. The accumulation of a film of organic matter upon submerged slides can be demonstrated by differential stains as well as by micro-chemical analyses. This hypothesis is supported by the fact that the favorable effect of solid surfaces is most striking in dilute nutrient solutions and by the fact that a large percentage of the bacteria multiply on the glass surface although many of them are not firmly attached to it.

Third, the solid surface, the capsular-like material which constitutes the holdfast of periphytic bacteria and the adsorbed organic matter may help retain the exo-enzymes near the bacteria until they can digest and ingest the nutrients. The interstices at the tangent of the bacterial cell and the glass surface may serve as concentration foci which retard the diffusion of the enzymes and the digestion end-products away from the cell. The inhibiting effect of continuous gentle agitation on the multiplication of bacteria in stored sea water is attributed to an acceleration in the diffusion of either the exo-enzymes or the digestion end-products away from the bacterial cells. It is generally recognized that bacterial adsorption to particles exerts a favorable influence on bacterial enzymatic activity.

Besides being of interest to students of bacterial physiology, this solid surface phenomenon is believed to be an important factor in explaining the vertical distribution of bacteria in the sea. In the ocean, bacteria are most abundant in the zone 10 to 50 meters below the surface where there are usually a few hundred or less bacteria per cc. Bacteria are only rarely encountered at depths exceeding 200 meters except on the sea floor where the bottom slime may contain millions of bacteria per cc. regardless of the depth of the overlying water. However, water collected from depths exceeding 200 meters contains sufficient nutrients to give rise to ten to a hundred million bacteria per cc. when the water is stored in small volumes. Why then is this deep water so sparsely populated? As ZoBell (1934) has shown, it cannot be attributed to the prevailing low temperature or high hydrostatic pressure. In view of the foregoing experimental observations, it is believed that the dearth of particulate matter to serve as solid surfaces is a factor which restricts the activity of bacteria in deep water.

SUMMARY

The storage of sea water is accompanied by a great increase in the number of bacteria and a decrease in bacterial species.

Appreciably more bacteria per cc. appear in small volumes of sea water than in large volumes of identical water.

Although the oxygen content of sea water is a factor which influences the activity of bacteria, it does not account for the denser bacterial populations which appear in the smaller volumes.

The favorable influence of small volumes is attributed to the contact of the water with the larger solid surface area in the small receptacles. Between ten and a hundred million bacteria per cc. have been demonstrated in sea water stored in sand which presents an enormous surface area whereas less than three hundred thousand bacteria per cc. appear in similar water stored in 10-liter bottles presenting relatively little surface area.

The beneficial effect of small volumes and their large surface areas occurs only in dilute nutrient solutions. Upon the addition of 10 to 100 mgm. of organic matter to sea water, the volume effects disappear.

Solid surfaces may serve as a resting place for periphytes, they may help concentrate the nutrients by adsorption or otherwise, or they may favor bacterial enzymatic activity and the absorption of metabolites.

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LITERATURE

Benecke, W., 1933. Bakteriologie des Meeres. *Abderhalden's Handb. der biol. Arbeitsmethoden,* **404**: 717.

Bond, R. M., 1933. A contribution to the study of the natural food-cycle in aquatic environments. *Bull. Bingham Oceangr. Col.,* **4**: 1.

Föyn, B., and H. H. Gran, 1928. Über Oxydation von organischen Stoffen im Meerwasser durch Bakterien. Avhand. *Utgitt av det Norske Videnskaps-Akad.,* 1 Oslo, No. 3.

Greenleaf, W. E., 1926. The influence of volume of culture media and cell proximity on the rate of reproduction of Infusoria. *Jour. Exper. Zoöl.,* **46**: 143.

Harvey, H. W., 1925. Oxidation in sea water. *Jour. Mar. Biol. Assoc.,* **13**: 953.

Henrici, A. T., 1933. Studies of freshwater bacteria. I. A direct microscopic technique. *Jour. Bact.,* **25**: 277.

Hitchens, A. P., 1921. Advantages of culture mediums containing small percentages of agar. *Jour. Infect. Dis.,* **29**: 390.

Keys, A., E. H. Christensen, and A. Krogh, 1935. The organic metabolism of sea-water with special reference to the ultimate food cycle in the sea. *Jour. Mar. Biol. Assoc.,* **20**: 181.

KROGH, A., 1931. Dissolved substances as food of aquatic organisms. *Rapports et Proces-Verbaux des Reunions,* **75**: 7.

LLOYD, B., 1930. Bacteria of the Clyde Sea area: a quantitative investigation. *Jour. Mar. Biol. Assoc.,* **16**: 879.

RAHN, O., 1932. Physiology of Bacteria. P. Blakiston's and Son, Philadelphia.

ROBERTSON, T. B., 1922. Reproduction in cell-communities. *Jour. Physiol.,* **56**: 404.

STEPHENSON, M., 1930. Bacterial Metabolism. Longmans, Green and Co., New York.

TANNER, F. W., AND D. L. SCHNEIDER, 1935. Effect of temperature of storage on bacteria in water samples. *Proc. Soc. Exper. Biol. and Med.,* **32**: 960.

WAKSMAN, S. A., H. W. REUSZER, C. L. CAREY, M. HOTCHKISS, AND C. E. RENN, 1933. Studies of the biology and chemistry of the Gulf of Maine. III. Bacteriological investigations of the sea water and marine bottoms. *Biol. Bull.,* **64**: 183.

WAKSMAN, S. A., AND C. L. CAREY, 1935*a*. Decomposition of organic matter in sea water by bacteria. I. Bacterial multiplication in stored sea water. *Jour. Bact.,* **29**: 531.

WAKSMAN, S. A., AND C. L. CAREY, 1935*b*. Decomposition of organic matter in sea water by bacteria. II. Influence of addition of organic substances upon bacterial activities. *Jour. Bact.,* **29**: 545.

WHIPPLE, G. C., 1901. Changes that take place in the bacterial contents of waters during transportation. *Tech. Quart.,* **14**: 21.

WINSLOW, C.-E. A., 1928. The Rise and Fall of Bacterial Populations. Jordan and Falk, " Newer Knowledge of Bacteriology and Immunology, University of Chicago Press, Chicago, p. 58.

WOODRUFF, L. L., 1911. The effect of excretion products of Paramecium on its rate of reproduction. *Jour. Exper. Zoöl.,* **10**: 557.

ZOBELL, C. E., 1934. Microbiological activities at low temperatures with particular reference to marine bacteria. *Quart. Rev. Biol.,* **9**: 460.

ZOBELL, C. E., AND E. C. ALLEN, 1933. Attachment of marine bacteria to submerged slides. *Proc. Soc. Exper. Biol. and Med.,* **30**: 1409.

ZOBELL, C. E., AND E. C. ALLEN, 1935. The significance of marine bacteria in the fouling of submerged surfaces. *Jour. Bact.,* **29**: 239.

ZOBELL, C. E., AND D. Q. ANDERSON, 1936. Vertical distribution of bacteria in marine sediments. *Bull. Am. Assoc. Petrol. Geol.,* **20**: 258.

ZOBELL, C. E., AND C. B. FELTHAM, 1934. Preliminary studies on the distribution and characteristics of marine bacteria. *Scripps Inst. Oceanog., Bull., tech. ser.,* **3**: 279.

THE UTILIZATION OF SOLUTES BY MOSQUITO LARVÆ

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Introduction

One of the important factors conditioning the suitability of a habitat for the development of mosquito larvæ is the available food supply. Chiefly for this reason, a number of investigators (see Hinman, 1930, for a summary of the literature) have studied the food requirements of mosquito larvæ. Since these insects usually ingest large amounts of particulate matter, it has been commonly concluded that this material is the principal, if not the only, source of food. However, in 1907, Pütter had shown that the chief food source for some aquatic organisms consists of organic colloids and solutes, and Matheson and Hinman (1930) suggested that such was probably the case with mosquito larvæ. Hinman (1930) presented evidence in support of this suggestion. He was able to rear small numbers of sterile *Ædes ægypti* in water very rich in organic matter that had been filtered through a Berkefeld N filter. Later (Hinman, 1932) he found that anopheline larvæ, in Berkefeld filtrates of the water of their natural habitat, could survive about 11 days and that some reached the second instar. If such water was subjected to dialysis against distilled water, and the dialysate sterilized by Berkefeld filtration, a medium was obtained containing only the solutes of the original water. In such a medium, sterile anopheline larvæ died within 3 days without reaching the second instar. Shipitsina (1930) obtained results similar to those of Hinman. In Berkefeld filtered water, anopheline larvæ made a slight growth, some reaching the second instar. She concluded that while organic colloids play some part in the nutrition of the larvæ, by far the larger rôle must be attributed to particulate matter.

Neither Hinman nor Shipitsina obtained any conclusive data concerning the ability of mosquito larvæ to utilize substances in true solution. The work upon this problem to be reported in this paper was made possible by the development (Trager, 1935*a*, *b*) of a reliable method of rearing mosquito larvæ free from microörganisms. Three different lines of evidence were obtained all supporting the view that mosquito larvæ can utilize solutes. First it was found that a growth factor required by the larvæ and present in Lilly liver extract No. 343 is water-soluble. Then a study of the effects of several substances of known chemical composition and known to exist in water in true solution showed that one of them, calcium chloride, is a substance essential for the normal development of the larvæ. Finally, it was possible to rear mosquito larvæ to the fourth instar in media ultrafiltered through collodion.

METHODS

Recently laid eggs of the yellow fever mosquito, *Ædes ægypti* (a stock colony of which was maintained), were freed from microörganisms by a 15-minute immersion in a fluid recommended by White (1931) and consisting of mercuric chloride 0.25 gram, sodium chloride 6.5 grams, hydrochloric acid 1.25 cc., ethyl alcohol 250 cc., and distilled water 750 cc. The eggs were handled in small glass " boats " (MacGregor, 1929) made from coverslips, were rinsed in sterile tap water, and inoculated into tubes containing an autoclaved 0.5 per cent solution of Lilly liver extract No. 343. The bacteria-free larvæ which subsequently hatched in this medium were washed in sterile distilled water and counted numbers of them were inoculated into the experimental tubes. For most of the work 18 × 160 mm. test tubes holding 6 cc. of medium were used. Each tube received three larvæ. The tubes were incubated at 28 ± 1° C. and were observed daily or every other day, the number of larvæ in each stage of development in each tube being noted. Figure 1 is a photograph of a typical culture tube in which one of the three larvæ has already pupated while the other two are in the fourth instar. Aseptic precautions were observed throughout, all the tubes were tested for sterility, and contaminated ones were discarded.

EVIDENCE THAT THE GROWTH FACTOR (*A*) EXISTS AS A SOLUTE

The first evidence that the larvæ could utilize substances in true solution was obtained in the course of the study of the properties of the accessory growth factor present in liver extract (factor *A*). In the presence of whole killed yeast and at concentrations of Lilly liver extract No. 343 below 0.5 per cent, the growth of *A. ægypti* larvæ is directly proportional to the concentration of liver extract and hence of factor *A* (Trager, 1936). There can thus be no doubt of the utilization of factor *A* by the larvæ. When a solution of liver extract 343 was dialyzed 24 hours in a collodion bag against an equal volume of distilled water, the concentration of *A* was as great in the outer as in the inner liquid. The factor thus dialyzes readily and must be present in true solution.

The Effects of Certain Solutes of Known Chemical Composition

Tubes were prepared each containing about 30–40 mg. powdered Harris vitamin B-free casein. They were sterilized by dry heat and to each tube was added the desired liquid medium, previously sterilized by autoclaving or by Berkefeld filtration. In tubes with casein and distilled water, first instar larvæ died within 2 to 3 days. The presence of 0.1 M glucose or sucrose did not prolong their life. However, with 0.1 M maltose several larvæ survived about a week. In the presence of a salt mixture containing NaCl, $CaCl_2$, $NaH_2PO_4 \cdot H_2O$ and K_2HPO_4 in a total concentration of 0.05 M, several larvæ survived over a week, while one reached the second instar and lived for 22 days. This would indicate an effect of the soluble salts on the longevity of small larvæ which were unable to grow because of the absence of accessory growth factors.

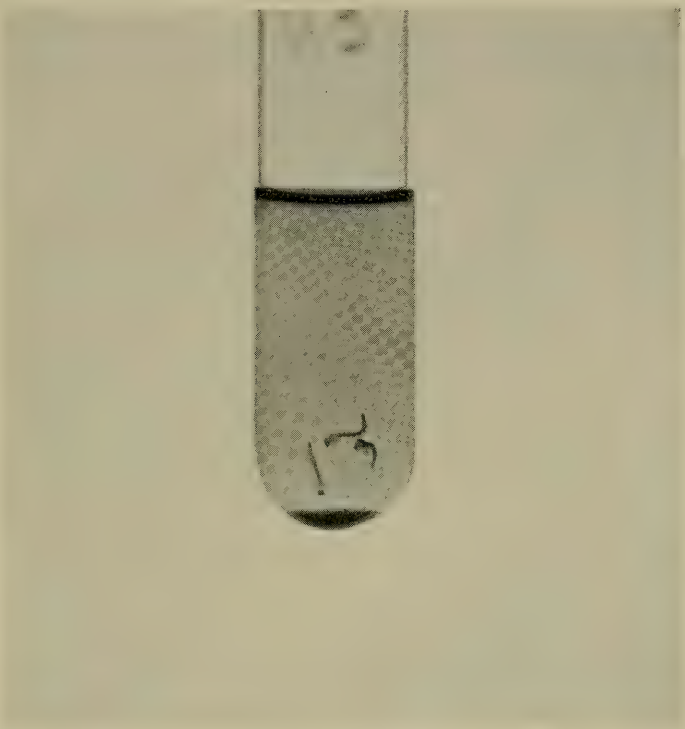

FIG. 1. Photograph of a tube containing casein, yeast extract, and liver extract. One of the three larvæ has pupated while the other two are in the fourth instar. Note the casein at the bottom of the tube. Photographed by J. A. Carlile.

Experiments were then performed to test the effect of solutions of salts and sugars on the growth of larvæ in the presence of accessory growth factors. It was previously shown (Trager, 1935*b*) that *A. ægypti* larvæ could be reared to maturity on a diet of casein, yeast extract (0.3 per cent yeast vitamin Harris) and liver extract 343. It was later found (Trager, 1936) that yeast extract contains the accessory growth factor present in liver extract (factor *A*) as well as a second accessory factor. It was accordingly possible to rear larvæ to maturity on a diet of sterile casein and autoclaved yeast extract. The minimum concentration of yeast vitamin Harris needed to supply enough factor *A* was 0.8 per cent. In such a medium, half of the larvæ inoculated would usually reach the adult stage within about 3 weeks.

This medium, consisting of casein and 0.8 per cent yeast vitamin Harris, was adopted as a basic medium and into it were incorporated various salts and sugars. Glucose, sucrose, and maltose in concentrations of 0.15, 0.10, and 0.05 M gave no better growth than that in the basic medium alone. The same was true of NaCl solutions in concentrations of 0.075, 0.050, and 0.025 M, as well as of various NaCl–KCl and NaCl–$MgSO_4$ mixtures. In all these media, on the sixteenth day, none of the larvæ had pupated and only about half had reached the fourth instar. But in the presence of both NaCl and $CaCl_2$ a striking acceleration of growth was noted. In a medium containing 0.04 M NaCl and 0.01 M $CaCl_2$ all of the larvæ reached the fourth instar in an average time of 8.5 days, two-thirds pupated in an average time of 12.5 days and gave normal adults [1] 2 days later. In a medium containing 0.045 M NaCl and 0.005 M $CaCl_2$ all the larvæ reached the fourth instar in an average time of 6 days, all pupated in an average time of 10.5 days and gave normal adults [1] 2 days later. The total time of development thus averaged 12.5 days, as compared to 9 days on a rich nutrient medium of whole dead yeast and liver extract. When other salts, or sugars, were substituted for part of the NaCl and $CaCl_2$ combination, growth was somewhat less than in the medium with 0.045 M NaCl and 0.005 M $CaCl_2$.

In another experiment a comparison was made of growth in the same basic medium in the absence of added salts and in the presence of concentrations of $CaCl_2$ ranging from 0.05 to 0.005 M. Growth in the presence of $CaCl_2$ was much more rapid than in its absence and was as good as in the presence of both NaCl and $CaCl_2$. These results show that $CaCl_2$, known to be in true solution in water, is an additional factor conditioning the growth of *A. ægypti* larvæ.

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[1] These adults were, however, smaller than those obtained in richer nutrient media.

Growth in Nutrient Media Containing Only Solutes

In previous work (1935*b*) it was found that *A. ægypti* larvæ grew quite normally in media consisting of yeast autolysate and liver extract, or yeast extract, casein digest and liver extract. Such media, sterilized by autoclaving, contained few particles but undoubtedly contained much material in colloidal suspension. From these results it is apparent that the insects can develop to the adult stage on a diet supplied either wholly in the form of colloids or partly as colloids and partly in true solution.

Several attempts were then made to rear the mosquito larvæ in media containing nothing but solutes. It had been found that fair growth could be obtained in autoclaved 2.5 per cent solutions of Vegex, a commercial yeast extract. These solutions after autoclaving contained some sediment. Three collodion bags were prepared each containing a fresh 5 per cent solution of Vegex and surrounded by an equal volume of distilled water. Both inner and outer liquids were covered with toluene and the dialysis was allowed to proceed 2 days at room temperature. The inner and outer liquids were then separated from the toluene, tubed, and autoclaved. No visible sediment formed in the dialysate after autoclaving. Each of two tubes of each solution was inoculated with three larvæ. The results follow.

Preparation 1.	Outer liquid.	One reached 3rd in 13 days, 4th in 22 days, none pupated.
	Inner liquid.	Three reached 3rd in 8.5 days, 2 reached 4th in 24 days, none pupated.
Preparation 2.	Outer liquid.	Two reached 3rd in 8 days, none reached 4th.
	Inner liquid.	Five reached 3rd in 10.5 days, 3 reached 4th in 23 days, 1 pupated in 35 days.
Preparation 3.	Outer liquid.	One reached 3rd in 10 days, 4th in 32 days, none pupated.
	Inner liquid.	Five reached 3rd in 9 days, 4 reached 4th in 29.5 days, none pupated.
Untreated 2.5 per cent Vegex.		Four reached 3rd in 6 days, 3 reached 4th in 12.5 days, 3 pupated in 19.5 days.

It is evident that in all three dialysates there was significant growth of the larvæ. It should be remarked that *all* the larvæ in these solutions reached the second instar and that the growth of some to the third and fourth instar did not take place as a result of cannibalism. In all cases there was more growth in the inner liquid than in the outer or dialysate. But the growth in the inner liquid was not as good as in untreated 2.5 per cent Vegex. Probably considerable amounts of nutrient material were adsorbed by the collodion membranes, which acquired a brown color.

A solution was prepared containing 1 per cent liver extract 343, 1 per cent Fairchild's peptone, and 2 per cent Difco yeast extract. Part of this was sterilized by filtration through a Berkefeld N filter. Another part was filtered under pressure through a disc-shaped collodion membrane held in a Seitz filter. The ultrafiltrate so obtained was sterilized by Berkefeld filtration. Each of the two liquids was tested alone and with casein, 6 larvæ being used for each test. The results were as follows.

Not ultrafiltered.	Six reached 3rd in 6.5 days, 2 reached 4th in 13 days, none pupated.
Same with casein.	Six reached 3rd in 4.5 days, 1 reached 4th in 12 days, none pupated.
Ultrafiltrate.	Three reached 3rd in 8.5 days, 2 reached 4th in 25 days, none pupated.
Same with casein.	Two reached 3rd in 9 days, 1 reached 4th in 13 days, none pupated.

Even in the solution filtered only through a Berkefeld and in the presence of casein, growth was not as good as in similar solutions sterilized by autoclaving. The Berkefeld candle probably adsorbed part of the required nutrients. Growth was not markedly affected by ultrafiltration or by the presence of solid protein in the form of casein. In another similar experiment a solution was prepared containing a fuller's earth extract (as a source of salts), 0.5 per cent liver extract 343, 1.2 per cent Vegex, and 2 per cent Fairchild's peptone. This was filtered under pressure through a collodion disc and was sterilized by Berkefeld filtration. Of 6 larvæ inoculated into this medium, 5 reached the third instar in 7.5 days and 2 reached the fourth in 14 days, but none pupated. The fourth instar larvæ in this, as in the preceding experiments, never attained the size of normal full-grown individuals.

DISCUSSION

The effect of calcium chloride on larval growth is, quite apart from its bearing on the utilization of solutes, of sufficient interest to merit some discussion. A certain amount of this salt is necessary for the normal development of *Ædes ægypti* larvæ. The effect is not an osmotic one. Sugars or other salts in equivalent concentrations do not show it.

Previous work on the effects of salts on mosquito larvæ has been concerned mainly with the resistance of various species to high salt concentrations. Wigglesworth (1933*c*) found that *A. ægypti* larvæ were killed by 1.1 per cent sodium chloride but, by very gradually evaporating the solution, could be made resistant to this concentration and to artificial sea water of a concentration osmotically equivalent to 1.75 per cent sodium chloride. He called attention to the fact that the larvæ can resist higher osmotic pressures in balanced salt solutions than in solutions of a single salt, and concluded that death of the larvæ in both cases was due, not to osmotic effects, but to the presence of directly toxic concentrations of certain ions. The possible stimulating effect of low concentrations of salts has received little attention. Roubaud, Colas-Belcour, and Treillard (1935), however, noted that while higher salt concentrations (in the form of sea water) were toxic to larvæ of three races of *Anopheles maculipennis,* lower concentrations, ranging from one-fifth to one-twentieth the strength of sea water, were distinctly favorable and resulted in better growth than that obtained in distilled water. They state that this effect may be due to a stimulation by the salts on bacterial growth or to the physico-chemical state of the water. In the light of my own results, it seems probable that the favorable effect of low sea water concentrations observed by these workers was the result of the specific action of essential ions.

It is, of course, well known that electrolytes have remarkable effects on a wide variety of animals and plants. One need only mention the pioneer work of Ringer (1880–82), Loeb (1910), and Osterhout (1922). Calcium ions seem to be of especial importance in many biological phenomena (see Bayliss, 1915) such as the coagulation of milk and blood, the beat of the heart, the transmission of nerve impulses, etc. Recently Warén (1933) has shown that calcium is essential for cell division in the plant *Micrasterias.* It is, therefore, not surprising that calcium should be a growth requirement of mosquito larvæ. Whether the calcium is essential for its own sake, or because of its antagonism toward some other ion, cannot be said at present.

The effects on larval growth of calcium chloride, known to exist in water as a solute, and of factor *A,* shown to be also in true solution, demonstrate clearly the ability of the larvæ to utilize substances in solution. Moreover, in view of the results described with ultrafiltrates, there can be no doubt that *Ædes ægypti* larvæ can grow to the last larval instar at the expense of nutrients existing wholly in true solution. It seems probable that the subnormal growth in such media containing only solutes is due, not to the inability of the larvæ to utilize nutrients in such a state, but to the experimental difficulty of providing, in the state of true solution, sufficiently high concentrations of some of the required substances. On the other hand, when solid food is available and the alimentary tract becomes packed with it, very high concentrations of soluble nutrients must be produced as a result of digestion. Such an explanation may very well apply to aquatic organisms other than mosquito larvæ. For example, the finding of Gellis and Clarke (1935) that *Daphnia magna* could grow in media containing colloids but not in media containing only solutes may be explained in this manner. The difference between nutrients existing in the environment as particles, colloids, or in true solution becomes a quantitative one. Whatever the initial state of the nutrient, it must be changed to a soluble form before it can be absorbed. When the nutrient exists as particles or even in colloidal form, the mosquito larvæ, or other aquatic organisms, are able to concentrate it by gathering up the particles. This they are not able to do with nutrients existing as solutes. These must enter the alimentary tract along with water and are present therein in the same concentration as in the surrounding fluid.

These considerations bring us to the problem of the method of ingestion of food by mosquito larvæ. The larval mouth brushes have been generally considered as an apparatus for gathering up fine particles. Shipitsina (1930) regarded them as filters capable of filtering out from the water even colloidal particles. She found that anopheline larvæ could filter out particles of colloidal silver with a diameter of 20 mμ but could not filter out the particles of soluble starch with a diameter of 5 mμ. Wigglesworth (1933*b*), using larvæ of *Ædes ægypti,* also concluded that very little fluid was ingested by mouth. He observed that the larva accumulates bits of food in its buccal cavity and does not swallow the food until a considerable bolus has accumulated. My own observations indicate that in particle-free media the brushes are probably of little use, but the swallowing movements of the larva enable it to ingest fluid.

Since larvæ can reach the fourth instar at the expense of nutrients in solution, these nutrients must somehow be absorbed by the larva. Howland (1930) has shown that dyes in solution in the external liquid can enter the alimentary tract of mosquito larvæ and be absorbed there. It seems most likely that soluble nutrients would follow the same path. However, Wigglesworth (1933*b*) found that, while the cuticle covering the body of mosquito larvæ is impermeable to water, water can and does regularly enter the body by way of the anal gills. The possibility of the entrance of nutrient solutes in this manner must be borne in mind, although Wigglesworth (1933*a*) has also shown that, while certain salts can enter the anal gill cells from the external medium, they cannot pass from the cells into the hemolymph.

SUMMARY

The larvæ of the yellow fever mosquito, *Ædes ægypti,* are able to utilize substances in true solution. They require for development at a normal rate a proper concentration of calcium chloride, known to exist in water as a solute. Their growth is likewise conditioned by an organic growth factor shown to be also a solute. Finally, the larvæ can develop at least as far as the fourth instar in media the nutrients of which exist entirely in true solution.

It gives me pleasure to acknowledge the helpful interest of Dr. R. W. Glaser.

REFERENCES

BAYLISS, W. M., 1915. Principles of General Physiology. Longmans, Green and Co. London.

GELLIS, S. S., AND G. L. CLARKE, 1935. Organic matter in dissolved and in colloidal form as food for Daphnia magna. *Physiol. Zoöl.,* **8**: 127.

HINMAN, E. H., 1930. A study of the food of mosquito larvæ (Culicidæ). *Am. Jour. Hyg.,* **12**: 238.

HINMAN, E. H., 1932. The rôle of solutes and colloids in the nutrition of anopheline larvæ. *Am. Jour. Trop. Med.,* **12**: 263.

HOWLAND, L. J., 1930. The nutrition of mosquito larvæ, with special reference to their algal food. *Bull. Ent. Res.,* **21**: 431.

LOEB, J., 1910. Ueber physiologische Ionenwirkungen, insbesondere die Bedeutung der Na-, Ca und K-Ionen. *Oppenheimer's Handbuch der Biochemie des Menschen und der Tiere,* Jena, **2**: 104.

MACGREGOR, M. E., 1929. The significance of the pH in the development of mosquito larvæ. *Parasitol.,* **21**: 132.

MATHESON, R., AND E. H. HINMAN, 1930. A seasonal study of the plancton of a spring-fed Chara pool versus that of a temporary to semi-permanent woodland pool in relation to mosquito breeding. *Am. Jour. Hyg.,* **11**: 174.

OSTERHOUT, W. J. V., 1922. Injury, Recovery and Death, in Relation to Conductivity and Permeability. J. B. Lippincott Co. Philadelphia.

PÜTTER, A., 1907. Die Ernährung der Wassertiere. *Zeitschr. f. allg. Physiol.,* **7**: 283.

RINGER, SYDNEY, 1880–82. Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle. *Jour. Physiol.,* **3**: 380.

ROUBAUD, E., J. COLAS-BELCOUR, AND M. TREILLARD, 1935. Influence de la concentration en sel marin sur le développement larvaire d'Anopheles maculipennis (var. atroparvus, fallax et labranchiæ). *Bull. Soc. Path. Exot.,* **28**: 568.

SHIPITSINA, G. K., 1930. The rôle of organic colloids in the nutrition of larvæ of Anopheles maculipennis. (In Russian.) *Bull. de l'Inst. rech. biol. de Perm,* **7**: 171.

TRAGER, W., 1935*a*. The culture of mosquito larvæ free from living microorganisms. *Am. Jour. Hyg.,* **22**: 18.

TRAGER, W., 1935*b*. On the nutritional requirements of mosquito larvæ (Ædes ægypti). *Am. Jour. Hyg.*, **22**: 475.

TRAGER, W., 1936. A growth factor required by mosquito larvæ. In press.

WARÉN, H., 1933. Über die Rolle des Calciums im Leben von Zelle auf Grund von Versuchen an Micrasterias. *Zeitschr. wiss. Biol. Abt. E, Planta,* **19**: 1.

WHITE, G. F., 1931. Production of sterile maggots for surgical use. *Jour. Parasitol.,* **18**: 133.

WIGGLESWORTH, V. B., 1933*a*. The effect of salts on the anal gills of the mosquito larva. *Jour. Exper. Biol.,* **10**: 1.

WIGGLESWORTH, V. B., 1933*b*. The function of the anal gills of the mosquito larva. *Jour. Exper. Biol.,* **10**: 16.

WIGGLESWORTH, V. B., 1933*c*. The adaptation of mosquito larvæ to salt water. *Jour. Exper. Biol.,* **10**: 27.

ENVIRONMENT AND SEX IN THE OVIPAROUS OYSTER OSTREA VIRGINICA

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The Virginia oyster is a member of that group of oviparous pelecypods in which each individual, with relatively few exceptions, is seasonally functional as either male or female, with the possibility of reversal to the opposite sex during the interval between two breeding seasons (Needler, 1932; Coe, 1932*a*, 1934; Burkenroad, 1936). All species of larviparous oysters so far as known have a more or less regularly alternating rhythm of male and female phases.

In the northern part of its range the Virginia oyster appears to be mainly or wholly protandric, while in somewhat warmer waters, at least as far southward as Virginia, a considerable proportion of the young oysters function as females during their first breeding season, which occurs at the age of one year. The more favorable the conditions are for the rapid growth of the young oyster, the more likely is the initial male phase to be omitted or aborted. Hence those localities and seasons in which the yearling oysters reach the largest mean size usually have the largest proportion of females in the first breeding season (Coe, 1934).

From North Carolina southward young oysters of both sexes reach functional maturity when only ten to twelve weeks of age.

In the older age groups the two sexes occur in approximately equal proportions, with some tendency toward an excess of males at the second breeding season and an excess of females in groups that are more than five years of age. It is unknown whether every individual changes its sex during even a long lifetime nor is it known whether more than a single change ever occurs.

The histological basis for these changes of sex depends upon the essential bisexuality of the gonads. In the young oyster before sexual maturity the gonad consists of a cortical layer of ovogonia surrounding a group of spermatogenic cells. In the typical case of protandry the spermatogonia proliferate rapidly, soon giving the gonad its dominantly male characteristics. Some ovogonia or ovocytes can nearly always be detected in the cortex, however. These, together with many sexually undifferentiated cells, remain as residual gonia after the functional male phase has been completed. The sexual phase for the ensuing year will then depend upon which of the two alternative types of cells are stimulated to renewed activity.

In the sexually immature young oyster which is to function as a female at its first breeding season, on the other hand, the proliferation of the spermatogonia in the primary bisexual gonad is soon checked and is succeeded by a differentiation and multiplication of ovogonia. The initial male phase is thus aborted, sometimes even after the appearance of a few spermatids.

In approximately one per cent of the yearling population neither type of sexuality is fully dominant, so that partial or complete hermaphroditism results. Self-fertilization is successful experimentally and presumably occurs occasionally in nature.

Seasonal protandry. Occasionally the gonad of the yearling hermaphrodite shows a typical ovarian structure except for groups of spermatogenic cells or ripe spermatozoa next the lumens of some of the follicles. In some of these cases the sperm mature and are discharged early in the breeding season, leaving the ovocytes and a few residual spermatozoa to be discharged later during the same summer, as is the case with the young of viviparous species (Coe, 1932*b*).

During the past few years evidence has been obtained which gives some indication as to the influence of environmental conditions in determining which of the two alternative types of cells in the primary bisexual gonad shall be stimulated to proliferation; that is to say, which of the alternative sexual phases shall be functional at the breeding season.

It has been previously shown (Coe, 1932*a*, 1934) that the mean size of the females in the yearling group from each locality considerably exceeds that of the males. In addition to the data previously presented, further evidence was obtained from the examination of the gonads of more than 3,000 individuals from the vicinity of New Haven, Connecticut, during their first breeding season, in 1935 and 1936.

Of 670 young oysters which had been collected in the harbor and transferred to deep water off the New Haven Breakwater there was a ratio of 7.66 females to each hundred males. The mean size of the females was 26.88 mm. as compared with 25.43 mm. for the males. Similar collections of 391 yearlings made at the same season from the natural beds at Milford, only about eight miles distant, gave a ratio of 24 females for each hundred males, the females at this locality having a mean length of 30.52 mm. and the males 30.24 mm. Tables I and II indicate the sex ratios which were found at several localities along the coast in the breeding seasons of 1932–1935, the entries listing immature individuals representing samples taken early in the season. The size of the shell at date of collection will obviously depend upon the length and favorableness of the two growing seasons in the young oyster's lifetime, but the size at the end of winter, at which time the sexual condition for the following season is presumably already determined, likewise shows an average larger size for females than for males.

TABLE I

Ostrea virginica. Sex at first breeding season. *Im* = sexually immature early in breeding season; *F* = female; *H* = hermaphroditic; *M* = male.

Locality	No. *Im.*	No. *M.*	No. *H.*	No. *F.*	Total	Percentage *F.*
New Haven (1932)	17	389	4	13	423	3.07
Great South Bay, L. I.	21	197	0	7	225	3.11
New Haven (1933)	1	129	0	7	137	5.11
New Haven (1935)	105	521	4	40	670	5.97
Milford (1935)	2	312	2	75	391	18.89
W. Sayville, L. I., (float)	0	154	4	48	206	23.30
Beaufort, N. C.	0	43	0	21	64	32.81

TABLE II

Ostrea virginica. Correlation of sex ratio and size. Mean length at first breeding season. *F* = female; *M* = male.

Locality	No. *M.*	Mean	No. *F.*	Mean	Ratio *F* to 100 *M*
		mm.		*mm.*	
New Haven (1932)	389	31.28	13	38.54	3.34
Great South Bay, L. I.	197	Ca. 29.	7	Ca. 34.	3.55
New Haven (1933)	129	39.90	7	44.14	5.42
New Haven (1935)	521	25.43	40	26.88	7.66
Milford (1935)	312	30.24	75	30.52	24.04
W. Sayville, L. I., (float)	154	46.33	48	59.33	31.17
Beaufort, N. C.	43	53.5	21	60.5	48.84

These results are in complete harmony with the evidence reported previously for other years and for other localities (Coe, 1932*a*, 1934). The data given in Tables I and II indicate that environmental conditions in the same locality may vary considerably from year to year, if it be assumed that samples of several hundred individuals are truly representative of the entire population.

Date of setting and length of growing season. Since the sex ratio of the yearlings is evidently correlated with the size at the end of winter as well as with the size at the first breeding season, it seems probable that the length as well as the favorableness of the growing season preceding the first winter may influence the proportion of females. Years in which much of the set occurs in early August presumably show a higher ratio of females among the yearlings of the following year than is the case when most of the set takes place in September, providing the environmental conditions are similar.

The time of sexual maturity of the yearlings also shows much variation in different years and at various localities (Table I). In the northern portion of the species' range a large proportion of the young remain sexually immature until their second year (Needler, 1932) but south of Cape Cod most of them become functional at some time during the summer at the end of their first year. It was previously reported (Coe, 1932*a*, 1934) that in 1932 most of the yearlings in the Woods Hole area postponed spawning until their second summer, but in more recent years nearly all have become functional before the end of the first breeding season.

Change of sex ratio during breeding season. The progressively increasing ratio of mature females in the yearling population as the season advances is shown by recent collections from Milford, Connecticut, obtained through the courtesy of Dr. Victor L. Loosanoff of the Bureau of Fisheries station at Milford. At the beginning of the breeding season, July 3, 1936, a sample containing 911 yearlings was composed of 77 sexually immature individuals, 780 ripe males, 10 hermaphrodites and 44 females. A second sample, taken August 13, at the height of the breeding season, consisted of 7 individuals that were still sexually immature, 410 males, 4 hermaphrodites and 58 females. A third sample near the end of the breeding season contained 92 males and 21 females, besides 176 individuals which had almost completely spawned out. For each hundred males there were 5.64 functional females in the first sample, 14.15 in the second and 22.83 in the third sample. The increased ratio of females resulted in part from the maturity of females which had not become functional at the beginning of the season and in part from individuals which would have been classed as hermaphroditic males earlier but have later transformed to females by the maturation of the spermatogenic cells and discharge of the resulting spermatozoa.

The breeding season occurs earlier on the oyster beds in shallow, warmer water but varies with the temperature from year to year. From one bed in Milford in July, 1935, the female ratio in a sample of 312 yearlings was 24.04, while from another bed in the same month of 1934 it was only 7.50. On July 28, 1936, 373 yearlings from New Haven harbor showed a female ratio of 12.54, as compared with 5.42 in 1933 and 7.66 in 1935.

The distinctly larger mean size of the females during the first breeding season is well illustrated by random samples consisting of 202 individuals taken from the culture floats at West Sayville. Of those having a shell length of more than 50 mm. there were 49 males and 42 females, while the group with a length of less than 50 mm. contained 105 males and only 6 females. If the selection had been made either by volume or by weight the superiority of the females would have been even more evident.

Tables I and II offer substantial evidence that there is a rather close correlation between the mean size of the individuals in the yearling population and the percentage of functional females, or the ratio of females to males. The mean size of the females in all cases exceeds that of the males and with few exceptions those years and those localities in which the metabolic conditions are most favorable, as indicated by the size attained at the end of the first year, have the largest proportions of females.

With this additional evidence it now seems fair to conclude that favorable metabolic conditions increase not only the mean size of the individuals but also the proportion of females in the yearling population.

Three assumptions may be suggested in explanation of this conclusion: (*a*) that inherent sex factors are associated with metabolic factors that lead to the more rapid growth of those individuals that are to become females; (*b*) that the more favorable conditions for growth stimulate the proliferation and growth of the ovogonia in the young bisexual gonad or inhibit the proliferation of the spermatogonia; (*c*) that there is in the immature oviparous oysters an inherent tendency toward an alternation of sexuality such as is usual in the viviparous species (Orton, 1926–27; Coe, 1932*b*).

The first two assumptions have been discussed in a previous paper (Coe, 1934). The last assumption would imply that those individuals that are to function as females in their first breeding season, when one year of age, may have already passed through an aborted protandric phase in the late autumn of the year of hatching, when only about four months old. There is some evidence that this assumption is justified, for it has been observed that the proliferation of spermatogonia and the incipient stages of spermatogenesis are most pronounced in the largest of the young individuals. It seems quite possible that these are the same individuals as those which function as females the following summer. The observed ratios in the various localities are in harmony with this supposition.

Protandry would thus dominate the sexuality of the oviparous species just as is the case in the viviparous forms (Coe, 1932*b*, 1934), the initial male phase being inaugurated, but aborted, in the autumn of the year of hatching for those individuals that function as females at the age of one year. If for any reason the protandric phase is delayed until the following spring, such individuals complete spermatogenesis and thus become functional males during the summer of the first breeding season. In those localities from Virginia northward which have the most favorable conditions for the inauguration of the aborted male phase in the autumn there will thus be the greatest proportion of females at the first breeding season.

It may be mentioned in conclusion that the primary gonads of both the oviparous and viviparous oysters show various grades of bisexuality, some individuals being more strongly masculine or more strongly feminine than others in appearance, as indicated by the relative abundance of the cells characteristic of the two sexes. This is likewise true of other pelecypods which normally experience one or more changes of sexual phase, as *Teredo* (Coe, 1935) and *Venus* (Loosanoff, 1936), as well as of certain gastropods, including *Patella* (Orton, 1928) and *Crepidula* (Coe, 1936). In all of these species the initial male phase is occasionally aborted, while other young individuals appear to be so strongly masculine that they have been designated as "true males." In the latter the female phase may be long delayed. Protandry is thus dominant in all the species mentioned but is not exclusive in any of them.

LITERATURE CITED

Burkenroad, Martin D., 1936. Rate of reversal of sexual phase in oviparous oysters. (In press.)

Coe, Wesley R., 1932*a*. Sexual phases in the American oyster (Ostrea virginica). *Biol. Bull.*, **63**: 419.

Coe, Wesley R., 1932*b*. Development of the gonads and the sequence of the sexual phases in the California oyster (Ostrea lurida). *Bull. Scripps Inst. Oceanog., Tech. Ser.*, **3**: 119.

Coe, Wesley R., 1934. Alternation of sexuality in oysters. *Am. Nat.*, **68**: 236.

Coe, Wesley R., 1935. Sequence of sexual phases in Teredo, Ostrea and Crepidula. *Anat. Rec.*, **64**: suppl., 81.

Coe, Wesley R., 1936. Sexual phases in Crepidula. *Jour. Exper. Zoöl.,* **72**: 455.

Loosanoff, Victor, 1936. Sexual phases in the quohog. *Science,* **83**: 287.

Needler, Alfreda B., 1932. American Atlantic oysters change their sex. *Prog. Rept. Atlantic Biol. Sta. and Fish. Exp. Sta.,* **5**: 3.

Orton, J. H., 1926–27. Observations and experiments on sex-change in the European oyster (O. edulis). *Jour. Mar. Biol. Ass.,* **14**: 967.

Orton, J. H., 1928. Observations on Patella vulgata. Part I. Sex-phenomena, breeding and shell-growth. *Jour. Mar. Biol. Ass.,* **15**: 851.

RENAL FUNCTION IN MARINE TELEOSTS

IV. The Excretion of Inorganic Phosphate in the Sculpin

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Our knowledge of the nature of the water and salt cycle in the marine teleosts has been markedly clarified by Smith (1930, 1932), who has demonstrated quite conclusively (1) that marine teleosts normally ingest considerable quantities of sea water, which subsequently undergo absorption in the gastro-intestinal tract; (2) that the greater part of the sodium, potassium, and chloride absorbed from the alimentary tract is excreted by some extra-renal route, presumably the gills; and (3) that essentially all of the absorbed magnesium, calcium, sulfate, and phosphate which leaves the body is excreted by the kidneys alone.

The renal excretion of inorganic phosphate in marine teleosts is particularly interesting. It has been shown by Marshall and Grafflin (1933) that the urine of the aglomerular goosefish (*Lophius piscatorius*) may contain large amounts of inorganic phosphate, which is presumably formed in the kidney from some as yet unknown precursor and secreted by the renal tubules. The basis for this interpretation is that injected inorganic phosphate is not excreted either by this fish or by the aglomerular toadfish (*Opsanus tau*). It is to be inferred from this fact that the renal tubules of the glomerular teleost, and perhaps of other animals, would be unable to secrete preformed inorganic phosphate, and that this substance would be excreted only by glomerular filtration. Observations upon frogs (Marshall and Grafflin, 1933) and dogs (Pitts, 1933) support this suggestion. There remains, nevertheless, the possibility that the tubules of the glomerular kidney might, like the tubules of the aglomerular kidney, be able to secrete inorganic phosphate from some occult precursor. Though no evidence in this direction has as yet been adduced for the frog or for mammals, the evidence presented in this paper clearly points to such an operation in the glomerular fish kidney.

Our present experiments were made upon the common sculpin (*My-*

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[1] I am indebted to Professor E. K. Marshall, Jr., for his constant advice and criticism during the present investigation, which was carried out at the Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine. I am also indebted to Professor H. W. Smith for critical comments and for assistance with the manuscript.

oxocephalus octodecimspinosus).[2] The study of phosphate excretion in this species has been found to be complicated by two factors: (1) the occurrence of a spontaneous increase in phosphate excretion under experimental conditions; (2) the frequent presence of a precipitate of magnesium phosphate in the urine. Discussion of the experiments upon the mechanism of phosphate excretion, in terms of glomerular and tubular activity, will be deferred until after the consideration of these two complicating factors.

METHODS

To determine the rate of urine flow, the bladder was first emptied, with the use of a fine glass catheter, by massage of the overlying abdominal wall. The urinary papilla was then tied off with fine silk thread; and subsequently, at the end of the collection period, the thread was cut and the urine removed by catheter. In this way repeated urine collections could be made upon the same animal. The region of the papilla was always thoroughly cleansed with distilled water and dried before catheterization to avoid contamination. In sacrifice experiments blood was obtained by syringe from the exposed heart (bulbus of aorta); in the course of an experiment it was obtained by syringe from the caudal vessels without exposure (see Grafflin, 1935*a*). Phosphate was determined by the method of Fiske and Subbarow (1925), and is uniformly expressed in terms of millimols per liter. Potassium oxalate was used as anticoagulant. Whenever the urine showed turbidity the precipitate was dissolved with a trace of glacial acetic acid before the phosphate content was determined. The determination of xylose was carried out upon Somogyi (1931) filtrates of plasma and urine. After absorption of the glucose on yeast, xylose was determined by the Folin (1929) modification of the Folin-Wu method. Xylose values are expressed as xylose, not as glucose equivalents, appropriate corrections being made for the diminished reducing power of xylose and its dilution by the water content of packed yeast (see Marshall and Grafflin, 1932).

OBSERVATIONS

Normal Urinary and Plasma Phosphate Concentrations in the Sculpin

Under normal conditions phosphate may appear in the urine of the sculpin in very high concentration, and it is not infrequent to observe a heavy urinary precipitate of magnesium acid phosphate (Pitts, 1934). This phosphate is entirely of metabolic origin, since phosphate is present in sea water in only slight traces. When oliguria is established by obstruction of the gastro-intestinal tract at the pyloric end of the stomach, phosphate continues to be excreted in high concentration (Grafflin and Ennis, 1934). (The oliguria is a consequence of the fact that the animal is dependent upon ingested sea water for its urinary water.) In the urine obtained from six sculpins immediately after catching on hook and line, Pitts (1934) observed phosphate values ranging from 50 to 155 mM./liter. In six additional freshly caught specimens we have observed values of 7, 40, 113, 130, 140, and 190 mM./liter. Plasma phosphate determinations on freshly caught sculpins gave values of 3.2, 3.4, 3.5, 3.8, 4.4, 4.5, and 5.6 mM./liter. It follows from these observations that phosphate may be highly concentrated, relatively to the plasma, in the urine of normal fish.

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[2] The nephron in this species consists of a glomerulus, neck segment, brush border segment (the homologue of the proximal convoluted tubule of higher forms) and initial collecting tubule; the brush border segment is bisegmental on cytological grounds (Defrise, 1932; Edwards, 1935).

Course of Urinary Phosphate Concentration in Sculpins with a Single Daily Catheterization

It was early observed, however, that when individual animals are followed from day to day, with a single catheterization and without tying of the urinary papilla, the urinary phosphate soon drops to low levels and remains there. For example, sculpin No. 14 was followed for twelve days: urinary PO_4 7.0, 20.6, 4.0, 0, 0, 0, 1.5, 0.3, 1.1, 1.7, 1.1, and 1.9 mM./liter. No. 20 was followed for six days: PO_4 149.0, 10.8, 3.6, 2.5, 1.4, and 0.3 mM./liter. The urine flow on the seventh day was 51 cc. per kilogram per 24 hours, PO_4 0.5 mM./liter. This fall in the urinary concentration of phosphate is in part due to diuresis, for it is known that, unless very special precautions are taken, sculpins readily become diuretic under experimental conditions (Grafflin, 1931, 1935*b*; Smith, 1932; Pitts, 1934; Grafflin and Ennis, 1934; Clarke, 1934).

Spontaneous Increase in Urinary Phosphate Concentration and Phosphate Excretion

However, the present investigation has demonstrated a further very significant fact which has been missed in previous studies; namely, that when urine samples are taken at short intervals from a sculpin previously undisturbed (for exceptions see below), the urinary phosphate concentration and the rate of phosphate excretion very often show a moderate or marked, but transitory, increase.[3] Data upon thirteen sculpins are given in Fig. 1. In eleven animals preliminary catheterization (open circles) was followed by a collection period (2 to 3.2 hours), with the urinary papilla tied. Since the phosphate values represent the average concentration for the period, the second points (solid circles) are plotted as of the mid-period. The other two animals (Nos. 70, 72) were catheterized twice without tying of the papilla, and the second points are plotted at the time of catheterization.

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[3] A similar rise was observed in the closely related daddy sculpin (*M. scorpius*), from 0.5 to 44 mM./liter in a short collection period, with the urinary papilla tied. It is to be noted that this species shows marked evidences of glomerular degeneration, some specimens being functionally entirely aglomerular (Grafflin, 1933).

A further group of eleven sculpins which were more extensively studied are recorded in Fig. 2. All of the animals, except No. 37, were studied by catheterization alone, and the actual times of catheterization are plotted (open circles). In the case of No. 37, three collection periods were run successively, and the mid-points of these periods are plotted. All sculpins recorded in Fig. 2 were followed on the second day, and five of them still further on the third day (Fig. 3).

The conditions under which the spontaneous increase in phosphate concentration was observed in these animals are varied.

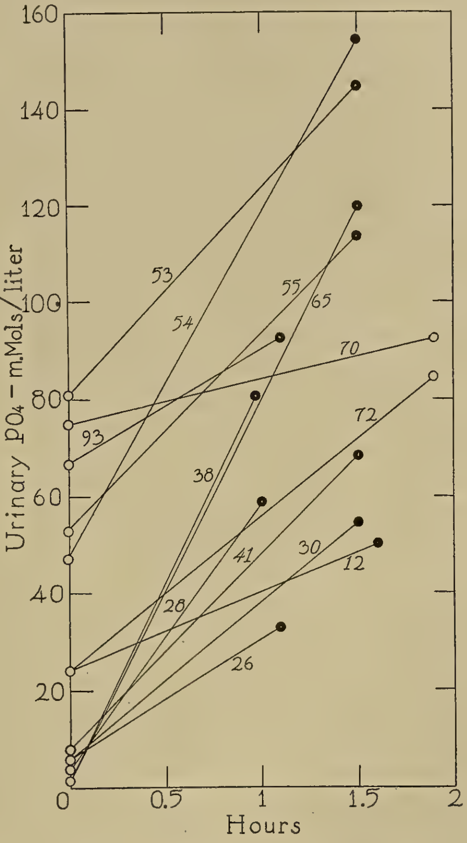

FIG. 1. Spontaneous increase in urinary phosphate concentration in the sculpin. Open circles, catheterization alone. Solid circles, urine collection period with urinary papilla tied.

Fig. 1.—In 11 specimens the rise occurred when the fish were first handled at varying intervals after catching: Nos. 70, 72—day after catching; No. 28—second day; No. 30—fourth day; No. 65—sixth day; Nos. 38, 41, 53, 54, 55, 93—interval indeterminate. (These latter fish were taken at random from the stock supply in a large live car. This stock was added to as fish were procured, and records of individual specimens were not kept.) No. 12 was catheterized once on the eleventh and eighteenth days and the rise was observed on the twenty-third day. No. 26 was catheterized once on the third, fourth, and fifth days, and the rise was observed on the sixth day.

Fig. 2.—In 8 specimens the rise occurred when the fish were first handled at varying intervals after catching: Nos. 47, 48, 49—day after catching; Nos. 43, 44, 45, 46—third day; No. 37—interval indeterminate (stock supply). Nos. 34 and 35 were catheterized once on the third day and the rise was observed on the fifth day. No. 36 was catheterized once on the third and fifth days and the rise was observed on the eighth day.

Figs. 1 to 3 deal only with the increase in urinary phosphate concentration, and a more detailed analysis of the experiments is given in Table I. From the nature of the data given, this table can only include experimental periods in which the urine flow was accurately determined, with the urinary papilla tied. Necessary explanatory considerations for the interpretation of this table are given at the top of p. 365.

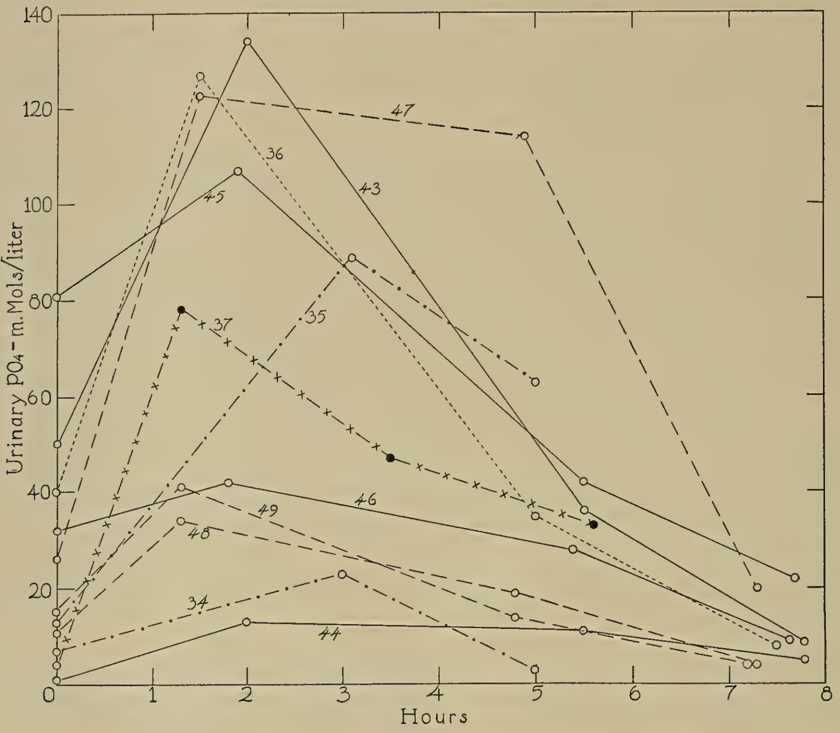

FIG. 2. Spontaneous increase in urinary phosphate concentration in the sculpin. Open circles, catheterization alone. Solid circles, urine collection period with urinary papilla tied. Groups of animals handled identically are graphed similarly (e.g., Nos. 43, 44, 45, 46; Nos. 47, 48, 49).

If, now, we proceed to analyze the data which we have obtained, we are led to the following considerations.

(1) Sculpins very often show a spontaneous increase in urinary phosphate concentration which may be slight, moderate or marked. It must be emphasized that in some specimens it is not observed at all. The degree of consistency with which it occurs is demonstrated by considering groups of sculpins with an identical history and handled identically under experimental conditions (see groups of animals similarly graphed in Fig. 2).

(2) In nineteen of the twenty-four sculpins recorded in Figs. 1 and 2 the spontaneous increase was observed when the animals were first handled, irrespective of the number of days they had been in captivity. The other five animals (Nos. 12, 26, 34, 35, 36) had been catheterized from one to three times previously (see above).

(3) The increase may occur irrespective of the initial concentration (first catheterization) over a wide range (1 to 81 mM./liter—Figs. 1 and 2).

(4) The increase is transitory, in general rising to a peak and then rather rapidly diminishing to low levels.

(5) It seems quite certain from the data available that the increase in urinary phosphate concentration is not associated with an increase in plasma phosphate concentration. Thus, in No. 15 (Table III), in which the urinary concentration showed a spontaneous increase in the first period and just as marked a decrease in the second period, the plasma concentration was considerably higher in the second period (4.2) than in the first (3.1). The rate of phosphate excretion was identical in the two periods. There seems to be no direct relationship between the absolute level of the plasma concentration and the concentration in the urine (see Table III). Three freshly caught sculpins examined at once gave the following plasma and urinary (in parentheses) phosphate values: 3.2 (190), 3.8 (130) and 4.4 (113) mM./liter.

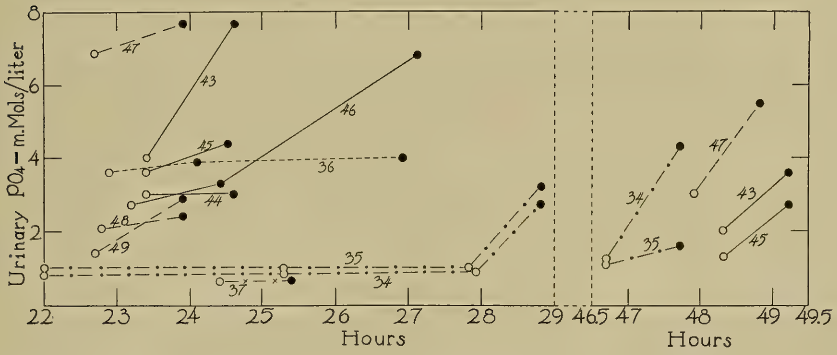

FIG. 3. Subsequent course of urinary phosphate concentration in sculpins recorded in Fig. 2. Open and solid circles, as in Fig. 2.

(6) As discussed above, the computed values for "Increase in PO_4 excretion" (Table I) are almost certainly minimal in most cases. A comparison of the figures for "Increase in PO_4 excretion" with those for "Increase in PO_4 concentration" shows wide discrepancies in the different animals. Obviously, an increase in concentration of 20 mM./liter, with a urine flow of 35 cc., would have exactly the same signif-

TABLE I

Data upon phosphate excretion during urine collection periods in sculpins reported in Figs. 1, 2 and 3.

Sculpin No.	Urine flow	Increase in PO_4 concentration	Additional PO_4 excreted[a]	Increase in PO_4 excretion[b]	PO_4 excretion[c]
	cc. per kg. per 24 hours	*mM./liter*	*mM.*	*mM. per kg. per 24 hours*	*mM. per kg. per 24 hours*
1. *Sculpins reported in Figs. 1 and 2 (No. 37 only)*					
12	7.0	26	0.005	0.18	0.35
26	32.7	27	0.014	0.88	1.08
28	15.4	56	0.015	0.86	0.91
30	16.7	49	0.022	0.82	0.92
37	10.0	74	0.033	0.74	0.78
38	17.2	79	0.043	1.36	1.39
41	26.6	61	0.024	1.62	1.84
53	8.3	64	0.020	0.53	1.20
54	6.8	108	0.027	0.73	1.05
55	9.4	61	0.015	0.57	1.07
65	10.0[d]	119	0.024	1.19	1.20
93	17.0[d]	26	0.008	0.44	1.58
2. *Sculpins on second day of handling (Fig. 3—22 to 29 hours)*					
34	40.7	>1.7	>0.0029	>0.07	0.11
35	49.8	>2.2	>0.0037	>0.11	0.16
36–1	33.2	0.3	0.0002	0.01	0.13
–2	113.6	0.1	0.0002	0.01	0.45
37	68.4	0	0	0	<0.07
43	72.0	3.7	0.0085	0.25	0.55
44	53.6	0	0	0	0.16
45	29.8	0.8	0.0006	0.02	0.13
46–1	61.8	0.6	0.0007	0.04	0.20
–2	73.1	3.5	0.0046	0.26	0.50
47	23.2	0.8	0.0005	0.02	0.18
48	41.5	0.3	0.0005	0.01	0.10
49	59.0	1.3	0.0022	0.08	0.16
3. *Sculpins on third day of handling (Fig. 3—46.5 to 49.5 hours)*					
34	93.4	3.3	0.0066	0.31	0.40
35	142.8	0.3	0.0008	0.04	0.20
43	83.5	1.6	0.0032	0.13	0.30
45	59.8	1.4	0.0015	0.08	0.16
47	11.4	2.5	0.0006	0.03	0.06

[a] Actual volume (in liters) of urine formed in collection period × increase in PO_4 concentration.
[b] Increase in PO_4 concentration × rate of urine flow.
[c] Actual PO_4 concentration of collection period urine (Figs. 1–3) × rate of urine flow.
[d] Approximate.

(1) "Increase in PO_4 excretion" is the difference between the observed excretion rate (last column) and the rate at which PO_4 would have been excreted if the urinary PO_4 concentration had not increased, but remained the same. Actually, the computed values may be considered as minimal values. The calculation is based on the assumption that the urine flow did not increase during the collection period, while in many cases, from the evidence available (Grafflin, 1931, 1935*b*, this paper; Pitts, 1934), it probably did.

(2) "Additional PO_4 excreted" represents the amount of PO_4 which would have to be added to the actual volume of urine formed in the collection period, in order to raise the PO_4 concentration from the level observed at the preliminary catheterization to that observed at the end of the collection period. In order to make clear the significance of this calculation, we must proceed at once to a discussion of the white precipitate frequently noted in sculpin urine. The following quotation is taken from Grafflin and Ennis (1934—p. 287): "It has been the experience of the workers at the Mount Desert Island Biological Laboratory over a period of years that the urine often shows a white precipitate; this is particularly true of the urine in freshly caught fish and of urine accumulated within the first 24 hours after catching. It has further been observed that a clear bladder urine may suddenly precipitate in the catheter or syringe when attempts are made to collect it; or again, the urine may be perfectly clear when collected, but subsequently develop a precipitate when allowed to stand at room temperature. The precipitate can always be dissolved by acidifying the urine with acetic acid." [4]

On analysis Pitts (1934) found the composition of this precipitate to correspond to $MgHPO_4 \cdot 3H_2O$. He makes the following statement: "It is possible that some of the variability of phosphate and magnesium excretion in these fish results from the ejection from the bladder of solid magnesium phosphate, excreted by the kidneys at some earlier time, but remaining in a precipitated state till a later collection period." If this were true, it might offer the entire explanation for the observed increase in urinary phosphate excretion. The significance of the calculation, "Additional PO_4 excreted," now becomes apparent. It represents simply the absolute magnitude of the hypothetical preformed store of phosphatic precipitate in the bladder (or elsewhere) which would be necessary to account for the increase in urinary phosphate concentration noted in the experimental periods.

icance, in terms of "Increase in PO_4 excretion," as an increase in concentration of 100 mM./liter, with a urine flow of 7 cc. Under the circumstances, it is evident that "Increase in PO_4 excretion" is really the fundamental consideration in a given experiment. Calculation from the data given in Section 1, Table I, shows that this quantity varies from 28 per cent to 99 per cent of the total "PO_4 excretion," with an average of 74 per cent.

(7) Since sculpins usually become progressively diuretic when handled repeatedly under experimental conditions, the total phosphate excretion may continue to increase tremendously when the concentration is steadily diminishing. This fact is strikingly demonstrated in the

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[4] This precipitate may occur in urines of widely varying PO_4 content. In the present study, on the one hand, cloudy urines (as withdrawn from the bladder) have been observed with a PO_4 concentration as low as 6 mM./liter. On the other hand, perfectly clear urines have been noted which contained as high as 100 mM./liter.

following tabulation (Table II) of the results obtained upon sculpin No. 37 (see Figs. 2 and 3).

TABLE II

Sculpin No. 37; 245 grams	Urinary PO_4	Urine flow	PO_4 excretion
	mM./liter	*cc. per kg. per 24 hours*	*mM. per kg. per 24 hours*
Pretying	4.0	(10.0) *	(0.04)
0–2.5 hours	78.0	10.0	0.78
2.5–4.6 hours	46.5	44.0	2.04
4.6–6.7 hours	32.8	77.4	2.54
24.4 hours (pretying)	1.0	—	—
24.4–26.4 hours	1.0	68.4	0.07

* Assumed maximum.

Causal Factors in the Spontaneous Increase in Phosphate Excretion

It will now be profitable to examine some of the factors inherent in the preceding experiments, in an attempt to determine whether one or more of them might be directly responsible for the observed spontaneous increase in phosphate excretion.

Tying of the Urinary Papilla; Catheterization.—Tying of the urinary papilla can be ruled out at once, since the increase occurs just as readily when sculpins are studied by catheterization alone as when the papilla is tied (Figs. 1 and 2). An evaluation of the factor of catheterization is more difficult. Sculpins are frequently very sensitive to this procedure and struggle vigorously, particularly when the papilla is catheterized for the first time. Usually, on subsequent catheterizations, little or no response is elicited. In certain experiments (Nos. 12, 26, 34, 35, 36—Figs. 1 and 2) the papilla had been dilated by one or more catheterizations previous to the day on which the increase was noted. The intensity of the stimulus in such animals is certainly much less than in animals catheterized for the first time, and one might be led to suppose that the procedure of catheterization is not of itself a significant factor in the observed rise. However, since this procedure is a constant one in the present experiments, it cannot be definitely ruled out.[5]

Pressure; Asphyxia.—That the pressure applied to the abdominal wall in milking the urine out of the bladder plays no rôle is demonstrated by experiments in which the urine was drained into the catheter by gravity alone, without the application of any pressure whatsoever (e.g. Nos. 70 and 72, Fig. 1). For the manipulations of catheterization and tying the fish were usually taken out of water. However, that a short period of asphyxia is of no significance in the observed rise is demonstrated by experiments with the animal under water and breathing normally (e.g. Nos. 30 and 41, Fig. 1).

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[5] The evidence is quite conclusive that in the sculpin catheterization is not followed by a shut-down in urine formation (Grafflin, 1935*b*). Even assuming that it did occur, calculation shows the impossibility of explaining the observed increase in PO_4 excretion solely on this basis.

Phosphatic Precipitate in Urine.—As previously discussed, sculpin urine frequently contains a white precipitate, which Pitts (1934) has shown to be $MgHPO_4 \cdot 3H_2O$. Pitts' suggestion that this precipitate may be retained in the bladder, and so lead to confusion in experiments upon phosphate excretion, has been quoted above. It is essential to determine whether retention of this precipitate, or the presence of phosphatic concretions, in the bladder or elsewhere might be responsible for the spontaneous increase in phosphate excretion which we have observed. With this end in view, the following experiments have been performed.

Bladder Washings.—In 2 sculpins the bladder was emptied by catheter and thoroughly washed out with isotonic salt solution acidified with acetic acid. The papilla was then tied and urine allowed to accumulate for 3 hours. Despite the bladder washing, the urinary PO_4 concentration showed an increase as compared with the pretying urine: No. 62, from 48 to 116 mM./liter; No. 63, from 75 to 100 mM./liter. The total PO_4 recovered from the bladder washings was 0.0015 mM. (No. 62) and 0.0008 mM. (No. 63). Evidence that even markedly turbid urine can be removed by catheter without leaving more than an insignificant trace of PO_4 in the bladder was obtained in 2 experiments on freshly caught sculpins. After preliminary emptying by catheter, the total PO_4 recovered from the bladder by washing as above was 0.0010 and 0.0015 mM.[6]

Ureteral Extractions.—In 3 sculpins the large ureteral trunk visible on the ventral surface of the kidney was dissected out and extracted separately for each animal with acetic acid. Total PO_4 recovery from trichloracetic acid filtrates of the extracts was in each case 0.0010 mM. or less.

Kidney Extractions.—From 4 sculpins the combined posterior and middle kidney was removed and extracted separately for each animal by grinding with washed sand and dilute acetic acid. The anterior portion of the kidney was not used as it is entirely lymphoid in nature. The kidney samples weighed 0.17, 0.25, 0.27, and 0.28 gram, and the total PO_4 recovered from trichloracetic acid filtrates of the extracts was 0.0020, 0.0021, 0.0031, and 0.0026 mM. Urine obtained just before the tissue samples were taken showed PO_4 concentrations of 64, 38, 82, and 93 mM./liter, respectively. Again, the combined posterior and middle kidney was removed from 4 freshly caught sculpins, and the entire sample (1.3 grams) extracted as before. Total PO_4 recovery from the filtrate was 0.0128 mM., giving an average of 0.0032 mM., comparable with the average (0.0025 mM.) of the 4 previous extractions.

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[6] In spite of the frequency with which magnesium phosphate is precipitated in sculpin urine, bladder calculi have never been observed. In 456 sculpins (used for another study—Grafflin and Gould, 1936) in which the bladder was opened, calculi were never encountered. To this number can be added several hundred specimens observed in the course of previous studies.

To evaluate these experiments, it is essential to know the order of magnitude of a preformed store of phosphate, either as the usual precipitate or in the form of concretions, which would be necessary to account for such increases in urinary phosphate excretion as we have observed. This calculation has been made for twelve of the animals in Figs. 1 and 2, and appears in Section 1, Table I, as " Additional PO_4 excreted." It varies from 0.005 to 0.043 mM., average 0.021 mM. This quantity is 17.5 times the average phosphate recovery from bladder washings (0.0012 mM.) in the above experiments. In the extreme case (No. 38) it is 36 times this latter quantity. The average hypothetical preformed store is at least 21 times the average recovery from ureteral extractions, and over 7 times that from 8 kidney samples (0.0029 mM.). This factor for the kidney extractions is deceptively low, since we must consider the phosphate contained in the blood, in the renal tissue cells, and in the urine contained in the lumina of the renal tubules and the collecting duct system.

The above experiments justify the conclusion that in the sculpin no significant retention of phosphate, as precipitated magnesium phosphate or as phosphatic concretions, occurs in the bladder, ureter or collecting duct system of the kidney. And we are permitted to conclude, in turn, (1) that the phosphate concentration of urine as withdrawn from the bladder can be accepted as the concentration of the urine as it is elaborated by the kidney; and (2) that the spontaneous increase in phosphate excretion reflects a real increase in renal activity.

In seeking still further for an explanation for the observed spontaneous increase in phosphate excretion, three possibilities were considered.

Muscular Activity.—Sculpins are normally very sluggish. When handled they frequently struggle quite vigorously, to such an extent that to hold them reliably in routine experimental work requires considerable experience. Phosphate compounds are of paramount importance in muscular activity, and muscular exercise in mammals regularly leads to increased urinary phosphate excretion. Under the circumstances, we feel that muscular activity offers the most likely explanation for the sharp increases which we have observed. The significant proportional increases in phosphate excretion observed on the second and third days of handling (Fig. 3, Table I) could be explained on the same basis, but at a much lower level of phosphate available for excretion.

Increased Absorption of Phosphate from the Alimentary Tract.—The phosphate of the body is derived essentially only from the food. Under experimental conditions sculpins develop a progressive diuresis, associated with a marked increase in the ingestion and gastro-intestinal absorption of sea water. A sudden increase in the absorption of sea water could well lead to rapid absorption of the phosphate contained in the partially digested food present, and so to a marked increase in phosphate excretion. That this mechanism is not fundamental in our experiments is indicated by the fact that some animals were starved for many days before the spontaneous increase was observed. However, it could conceivably play a rôle in animals handled soon after catching.

Reflex Effects upon the Kidney.—While tying of the papilla plays no rôle, reflex effects from the stimulus of catheterization cannot be definitely ruled out as a factor. It is impossible to rule out reflex effects from the handling of the skin. Many injection experiments have shown that liberation of adrenalin in the excitement of experimental handling would not of itself lead to increased phosphate excretion.

The Question of Tubular Secretion of Phosphate in the Sculpin

In approaching the question of tubular secretion, we were guided by the belief of Jolliffe, Shannon and Smith (1932) and Clarke and Smith (1932) that the xylose clearance could be used as a measurement of the rate of glomerular filtration. More recent experiments (Richards, Westfall and Bott, 1934; Shannon, 1934, 1935*a*, 1935*b*; Shannon and Smith, 1935; Van Slyke, Hiller and Miller, 1935*a*, 1935*b*) have shown that there is some reabsorption of xylose by the renal tubules, and that the rate of glomerular filtration can apparently be quite accurately measured in the dogfish, dog and man by the use of inulin. Clarke (1936) has found in the sculpin that the xylose clearance is about 20 per cent less than the simultaneous inulin clearance. But this discrepancy is not of such an order of magnitude as to invalidate the experiments which we had done with xylose in this animal. Our experiments consist of a series of simultaneous phosphate and xylose clearances, as given in Table III. (Clearance $= (U/P)V$, i.e., urine/plasma ratio times urine flow.) Data on the total phosphate excretion in these experiments are included in this table for comparison with the data in Table I. The tubular clearance of phosphate has been calculated by deducting the xylose clearance × 1.25 (in accordance with Clarke's figures) from the observed phosphate clearance. In a series of observations on normal sculpins with low urine flow, the maximal U/P ratio for xylose observed by Clarke (1934) was 6.5. In our early experiments plasma and urine were removed from three sculpins immediately after catching, and the phosphate U/P ratios were 59, 34 and 26. These data would indicate that the U/P ratio for phosphate in the sculpin may normally be considerably greater than can be accounted for by the reabsorption of water from the glomerular filtrate. This indication is established as a fact by the data in Table III, where in four experiments direct xylose-phosphate comparisons were carried out. In only one instance did the phosphate clearance approximate the glomerular clearance, after allowing for 20 per cent reabsorption of xylose.[7] A representative protocol for this series of experiments follows.

Sculpin No. 15; 330 grams. July 17. From live car stock at noon; urine PO_4 24 mM./liter. 5:30 P.M., 0.4 gram xylose intramusc. July 18. 8:17 A.M., urine PO_4 13 mM./liter; bladder emptied, papilla tied. 10:13, bled 1.5 cc. 12:09 P.M., urine 0.35 cc.; papilla retied. 2:05, bled 1.5 cc. 4:01, urine 1.25 cc.

TABLE III

Xylose—inorganic phosphate data for the sculpin.

Sculpin No.	Duration of Period	Urine Flow	Plasma		$\frac{U}{P}V$		$\frac{\text{Phosphate}}{\text{Xylose}}$	Tubular Clearance of Phosphate	Total Phosphate Excretion
			Xylose	Phosphate	Xylose	Phosphate			
	hours	*cc. per kg. per 24 hours*	*mg. per cent*	*mM./liter*	*cc. per kg. per 24 hours*			*cc. per kg. per 24 hours*	*mM. per kg. per 24 hours*
10	5.1	42.6	229	2.7	41.3	2096	50.8	2044	5.67
11	5.2	15.8	261	4.0	15.6	337	21.6	318	1.34
15	3.9	6.5	222	3.1	37.7	103	2.7	56	0.32
	3.9	23.3	183	4.2	53.6	75	1.4	8	0.33
17	4.1	8.6	142	2.7	8.6	409	46.4	398	1.10
	4.1	21.5	102	2.7	32.3	957	29.6	917	2.58

The frequency and the magnitude of the observed spontaneous increase in urinary phosphate concentration and excretion make it clear that experiments upon the excretion of exogenous phosphate would be uninterpretable until any significant increase could be adequately ruled out in a given experimental animal. It seems reasonable to suppose that in certain of the animals reported earlier (Nos. 12, 26, 34, 35, 36), catheterized at long intervals, the spontaneous rise may have occurred more than once. The question arose as to whether any significant increase could be suppressed, and the total rate of excretion of endogenous phosphate could be forced to low levels, if a sculpin were followed closely (i.e., persistently, with repeated catheterizations not separated by long intervals) over a sufficiently long period. Lack of space prohibits a detailed discussion of this aspect of the problem, but the summarized data in Figs. 2 and 3 and Table I indicate that such a condition can be achieved in the sculpin, and that such an animal could be used with confidence in studying the behavior of the kidney toward exogenous phosphate.

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[7] It is to be noted that when the sculpin kidney is rendered functionally aglomerular by heavy phlorizinization, phosphate continues to appear in the urine (Marshall and Grafflin, 1932).

In summarizing our observations, we may contrast the aglomerular kidney of the goosefish and toadfish, as studied by Marshall and Grafflin (1933), with the glomerular kidney of the sculpin, as examined here. Both types of kidney excrete inorganic phosphate; the one, where there is no glomerular filtration, the other at U/P ratios so much greater than the simultaneous U/P ratios of xylose that there is no doubt but that tubular activity is involved. The precursor from which the urinary inorganic phosphate is derived in the aglomerular goosefish and toadfish is unidentified, but it is certainly not inorganic phosphate in the plasma. By inference, until evidence to the contrary is adduced, it may be assumed that the tubular secretion of phosphate in the glomerular kidney is an homologous process. Whether the tubules of the glomerular fish kidney can secrete preformed inorganic phosphate cannot be answered from the present data.

The demonstration that the tubules of the glomerular fish kidney can secrete phosphate, presumptively derived from some unidentified precursor in the plasma other than inorganic phosphate, raises the question whether a similar process can occur in the frog or even in the mammal. We know of no evidence at the present time to enable us to answer this question.

SUMMARY

1. When urine samples are taken at short intervals, the urinary phosphate concentration and excretion in the sculpin very often shows a moderate or marked, but transitory, increase. It is demonstrated that this transient increase is not an artifact due to phosphate precipitates in the bladder, ureter or collecting ducts.

2. By repeated catheterization the rate of endogenous phosphate excretion in the sculpin can be reduced to a low level, and any significant spontaneous increase in excretion can be suppressed.

3. It is shown, by a comparison of simultaneous xylose and phosphate clearances, that the glomerular kidney of the sculpin can under certain conditions excrete a urine with a phosphate concentration in excess of that which could be explained by glomerular filtration.

4. In line with Marshall and Grafflin's observations that the aglom-

erular kidney excretes phosphate that is derived from some unidentified precursor (other than inorganic phosphate in the plasma), the present observations establish the presumption that a similar process occurs in the glomerular kidney of the sculpin.

LITERATURE CITED

Clarke, R. W., 1934. *Jour. Cell. and Comp. Physiol.,* **5**: 73.

Clarke, R. W., 1936. *Bull. Mt. Desert Island Biol. Lab.,* p. 25.

Clarke, R. W., and H. W. Smith, 1932. *Jour. Cell. and Comp. Physiol.,* **1**: 131.

Defrise, A., 1932. *Anat. Rec.,* **54**: 185.

Edwards, J. G., 1935. *Anat. Rec.,* **63**: 263.

Fiske, C. H., and Y. Subbarow, 1925. *Jour. Biol. Chem.,* **66**: 375.

Folin, O., 1929. *Jour. Biol. Chem.,* **82**: 83.

Grafflin, A. L., 1931. *Am. Jour. Physiol.,* **97**: 602.

Grafflin, A. L., 1933. *Anat. Rec.,* **57**: 59.

Grafflin, A. L., 1935*a*. *Biol. Bull.,* **69**: 245.

Grafflin, A. L., 1935*b*. *Biol. Bull.,* **69**: 391.

Grafflin, A. L., and D. Ennis, 1934. *Jour. Cell and Comp. Physiol.,* **4**: 283.

Grafflin, A. L., and R. G. Gould, Jr., 1936. *Biol. Bull.,* **70**: 16.

Jolliffe, N., J. A. Shannon, and H. W. Smith, 1932. *Am. Jour. Physiol.,* **100**: 301.

Marshall, E. K., Jr., and A. L. Grafflin, 1932. *Jour. Cell. and Comp. Physiol.,* **1**: 161.

Marshall, E. K., Jr., and A. L. Grafflin, 1933. *Proc. Soc. Exp. Biol. and Med.,* **31**: 44.

Pitts, R. F., 1933. *Am. Jour. Physiol.,* **106**: 1.

Pitts, R. F., 1934. *Jour. Cell. and Comp. Physiol.,* **4**: 389.

Richards, A. N., B. B. Westfall, and P. A. Bott, 1934. *Proc. Soc. Exp. Biol. and Med.,* **32**: 73.

Shannon, J. A., 1934. *Jour. Cell. and Comp. Physiol.,* **5**: 301.

Shannon, J. A., 1935*a*. *Am. Jour. Physiol.,* **112**: 405.

Shannon, J. A., 1935*b*. *Jour. Clin. Invest.,* **14**: 403.

Shannon, J. A., and H. W. Smith, 1935. *Jour. Clin. Invest.,* **14**: 393.

Smith, H. W., 1930. *Am. Jour. Physiol.,* **93**: 480.

Smith, H. W., 1932. *Quart. Rev. Biol.,* **7**: 1.

Somogyi, M., 1931. *Jour. Biol. Chem.,* **90**: 725.

Van Slyke, D. D., A. Hiller, and B. F. Miller, 1935*a*. *Am. Jour. Physiol.,* **113**: 611.

Van Slyke, D. D., A. Hiller, and B. F. Miller, 1935*b*. *Am. Jour. Physiol.,* **113**: 629.

SOME UNUSUAL CYTOLOGICAL PHENOMENA IN THE SPERMATOGENESIS OF A HAPLOID PARTHENOGENETIC HYMENOPTERAN, ÆNOPLEX SMITHII (PACKARD)

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Since the early part of the century numerous investigators have been concerned with the cytology of haploid animals. For extensive bibliographies pertinent to the cytology of haploid forms the reader is referred to publications by A. Vandel (1931), Franz Schrader and Sally Hughes-Schrader (1931), and Ann R. Sanderson (1933).

More recently Magnhild Torvik-Greb (1935) made a special study of the chromosomes of *Habrobracon juglandis* and also showed that the spermatogenesis of haploid males and diploid biparental males follows the same course. Allan C. Scott (1936) reported haploidy in the males of a coleopteran, *Micromalthus debilis,* and especially emphasized aberrant phenomena associated with spermatogenesis.

The development of the germ cells of the various haploid metazoan groups is much the same. Obviously a convergence or a parallelism is to be expected in view of the fact that no reduction of chromatin is necessary. The salient and conventional features identified with spermatogenesis have been pointed out by many authors. In the first spermatocyte there may be an attempt to recapitulate the reduction division, which is always abortive as regards chromosomes, or the division may be omitted entirely. When division of the cell does take place it is in the form of the pinching off of a non-nucleated cytoplasmic bud. The second spermatocyte division ordinarily is equational, producing two functional spermatids. However, as in the case of *Apis mellifica* (Meves, 1907), the second spermatocyte division may be unequal, producing only one functional spermatid.

Although haploid studies have been concerned chiefly with testing the validity of the concept that males are produced from unfertilized eggs and in confirming the usual development, recently a certain rather unique phenomenon has been reported. Allan E. Scott (1936) found, in haploid parthenogenetically produced males of *Micromalthus debilis,* that a unipolar spindle was regularly produced in the first spermatocyte division. During the first spermatocyte anaphase apparently the chromosomes move away from the unipolar spindle, the movement being independent of the half spindle fibers. The popular idea that spindle fibers are responsible for chromosomal movement is thus shown to be deficient.

It is the purpose of the present paper to describe the spermatogenesis of *Ænoplex smithii.* Emphasis will be placed on unusual cytological aspects which may contribute to our knowledge of the origin of half spindle components and to the morphological and functional reality of half spindle fibers.

Materials and Methods

Ænoplex smithii is the principal ichneumonid hyper-parasite of *Spilocryptus extrematus,* the latter living parasitically on the larvæ of *Samia cecropia,* a moth. The males develop only from unfertilized eggs and are thus produced parthenogenetically.

The original material for this investigation was collected in the vicinity of Chicago, Illinois. The data presented in this paper, however, are based entirely on laboratory specimens reared from virgin females which lay only male-producing eggs.

Male gonads of late pupæ proved to be the most satisfactory for cytological study, showing few gonial and numerous spermatocyte cells. The gonads of females used to secure chromosome counts in the gonial stage were taken from early pupæ. Gonads were dissected out in water and immediately transferred to a fixative, Bouin's fluid being found most favorable. Gonads were dehydrated and cleared in the usual manner, imbedded in paraffin and sectioned at six microns in thickness.

Heidenhain's iron hæmatoxylin (long method) was employed and destaining was done with a saturated aqueous solution of picric acid. When destained sufficiently the sections were exposed briefly to the fumes of concentrated ammonium hydroxide, dehydrated, cleared in xylol and mounted.

The gonads of more than one hundred males treated according to the above method were used as a basis for this study.

Spermatogonia and Chromosome Number of Haploid Males

The paired testes lie dorsal to the intestine in the posterior half of the abdomen. Internally the testes are divided into many cysts, containing cells in the various stages of development.

Testes of very early pupæ show cysts containing only spermatogonial cells. The cells are arranged in the cyst so as to give a compact, rosette appearance. Divisions in gonial cells are few, but are synchronous in each cyst.

It was only from spermatogonial division figures that it was possible to count the chromosomes. The number was established as thirteen (Fig. 1), representing the haploid number of this species.

Chromosome Number of Diploid Females

Polar views of the metaphase of gonial cells taken from late female pupæ show twenty-six chromosomes (Fig. 2). Therefore it is established cytologically that females are diploid and that the males developing from eggs laid by virgin females are haploid.

Spermatocytes and the Maturation Divisions

The testes of late pupæ show few gonial cells but show many first spermatocytes, second spermatocytes, spermatids, and spermatozoa. The first spermatocytes at the end of the growth period form a loose rosette or are scattered indifferently within the cyst. They are considerably larger than late gonial cells. The nucleus of the spherical first spermatocyte (Fig. 3) is prominent and appears reticulated, having one or more poorly defined nucleoli. A prominent cytoplasmic inclusion, which has been referred to frequently as the " Zellkoppel " or " interzonal body," is present in these cells.

In the late prophase the nuclear membrane becomes indistinct and the chromatin material becomes resolved into chromosomes (Fig. 4). At a slightly later stage the spherical spermatocyte has elongated (Fig. 5), the nuclear membrane has disappeared and the chromosomes have massed. A constriction has appeared, partially isolating that portion of the cytoplasm which subsequently will be pinched off as a non-nucleated bud. Indistinct fibers are seen extending from the vicinity of the chromosomal mass into the bud. This stage probably simulates the metaphase of an ordinary mitosis or meiosis. As a rule the bud contains the " interzonal body " (Fig. 9) which is not visible in some of the eosin counterstained preparations (Fig. 6). When the bud is almost completely detached, distinct fibers can be seen extending between it and the nucleated mother cell (Fig. 6). Simultaneously with the pinching off of the bud there arises a monopolar spindle (unipolar or half spindle), the components of which would appear to be formed as outgrowths from the massed chromosomes. The monopolar spindle is cone-shaped with the apex pointing toward the bud; although at first small and inconspicuous, it later becomes progressively larger, elongates and becomes very prominent (Fig. 7).

The massed chromosomes and their attached half spindle components next separate into two approximately equal halves (Figs. 8–9).

Simultaneously with this separation and with the spindle elongation there appears to be distinct anaphasic movement on the part of the chromosomes. At the completion of this anaphasic movement the chromosomes, with the attached spindle fibers trailing, have traversed the cell and occupy that part of the cell distal to the locus of the formerly attached bud (Fig. 10).

There is no formation of a nuclear membrane between the first and second maturation divisions. The telophase chromosomes gather into a dense compact mass during an abbreviated interkinesis. In the formation of this interkinetic mass the chromosomes, preceding, apparently drag their attached fibers with them (Fig. 11), so that in early interkinetic masses the fibers of the monopolar spindle radiate from the mass (Fig. 12). In later interkinesis the spindle fibers completely disappear (Fig. 13).

New spindle fibers identified with the second spermatocyte division soon make their appearance. The fibers, a number of which constitute the bipolar spindle, from all appearances are formed as outgrowths from some material contained within the interkinetic chromosomal mass. The components of each half spindle comprising the bipolar spindle are at first darkly staining short extensions from the chromosomal mass (Fig. 14) but subsequently are less basophilic and become progressively longer (Fig. 15). The components of each half spindle very early assume a cone formation. The cones, however, do not grow out in opposite direc-

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EXPLANATION OF PLATES

All figures are camera lucida drawings drawn to the same scale. They were originally drawn to represent a magnification of 4,807 and have been reduced one-third as represented here.

Explanation of Plate I

Fig. 1. Side view of spermatogonial metaphase showing thirteen chromosomes, the haploid number.

Fig. 2. Polar view of female gonial cell showing twenty-six chromosomes, the diploid number.

Figs. 3–4. First spermatocytes, early and late prophase respectively.

Figs. 5–6. First spermatocytes showing the pinching off of the cytoplasmic bud and the formation of the monopolar spindle.

Fig. 7. Second spermatocyte showing prominent monopolar spindle.

Figs. 8–10. Second spermatocyte showing anaphasic movement of the chromosomes, with spindle fibers trailing.

Fig. 11. Chromosomes of second spermatocyte, with spindle fibers trailing, gathering to form interkinetic mass.

Fig. 12. Second spermatocyte with half spindle fibers radiating from interkinetic mass.

Fig. 13. Interkinetic mass. Half spindle fibers have disappeared.

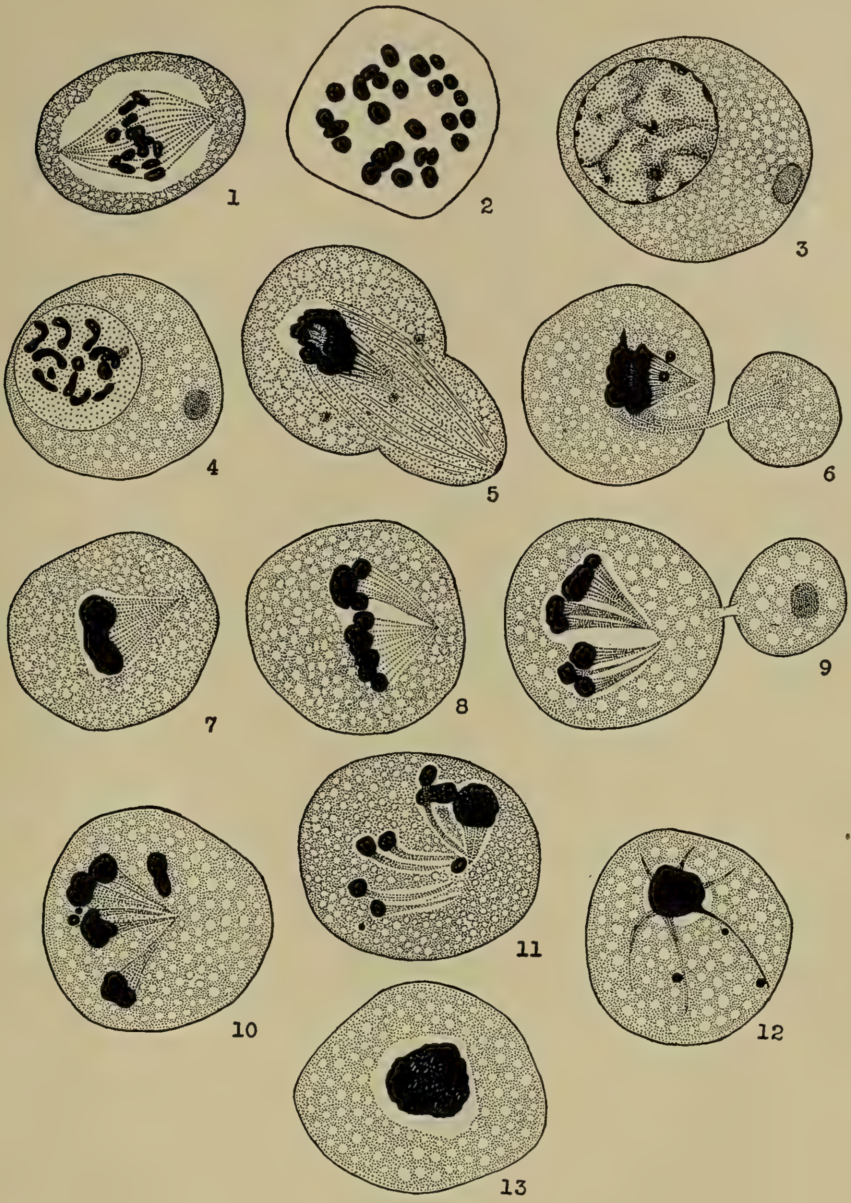
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Plate I

tions in the same plane. On the contrary, both cones not infrequently appear to grow out in the same general direction and in forming the bipolar spindle in which the two halves are opposed in the same plane (Fig. 17), each half cone may be generally figured as describing an arc of about ninety degrees. In the course of such movement many crescentic figures may be seen (Figs. 14–16).

THE SPERMATIDS

The second spermatocyte division may be equal, producing two normal spermatids (Figs. 18–19). Frequently, however, when the division has proceeded to late anaphase, it is suppressed (Fig. 22) and the cytoplasm of the two potential spermatids, instead of separating, fuses and an aberrant spermatid is formed. This spermatid is twice the size of the normal spermatid and is at first binucleate (Figs. 23–25). Later the two nuclei of the aberrant spermatid fuse forming a single large nucleus (Fig. 26).

In early spermiogenesis the metamorphosis of the normal spermatids (Figs. 20–21) and the aberrant spermatids (Figs. 27–28), both types of which may be found within the same cyst, appears to be similar except for the distinct dimorphism in size. Dimorphism in size of sperm has not been observed and therefore no evidence is available that would suggest that these large spermatids metamorphose into functional spermatozoa. It would seem likely that they are pathological in character.

UNUSUAL CYTOLOGICAL PHENOMENA AND POSSIBLE SIGNIFICANCE

Monopolar Spindle

Spindles which normally have only one pole have been reported recently by several authors. Metz (1933) described a monocentric mitosis which normally effects a regular and precise segregation of chromosomes in the first spermatocyte in *Sciara*. Sally Hughes-Schrader

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EXPLANATION OF PLATE II

FIG. 14. Origin of bipolar spindle from interkinetic chromosomal mass of second spermatocyte.

FIGS. 15–16. Crescent figures of second spermatocyte prophase.

FIG. 17. Metaphase of second spermatocyte division.

FIGS. 18–19. Normal spermatids.

FIGS. 20–21. Early spermiogenesis of normal spermatids.

FIGS. 22–25. Binucleated aberrant spermatids.

FIG. 26. Large mononucleated spermatid, the nucleus being formed by the fusion of two nuclei.

FIGS. 27–28. Early spermiogenesis of aberrant spermatid.

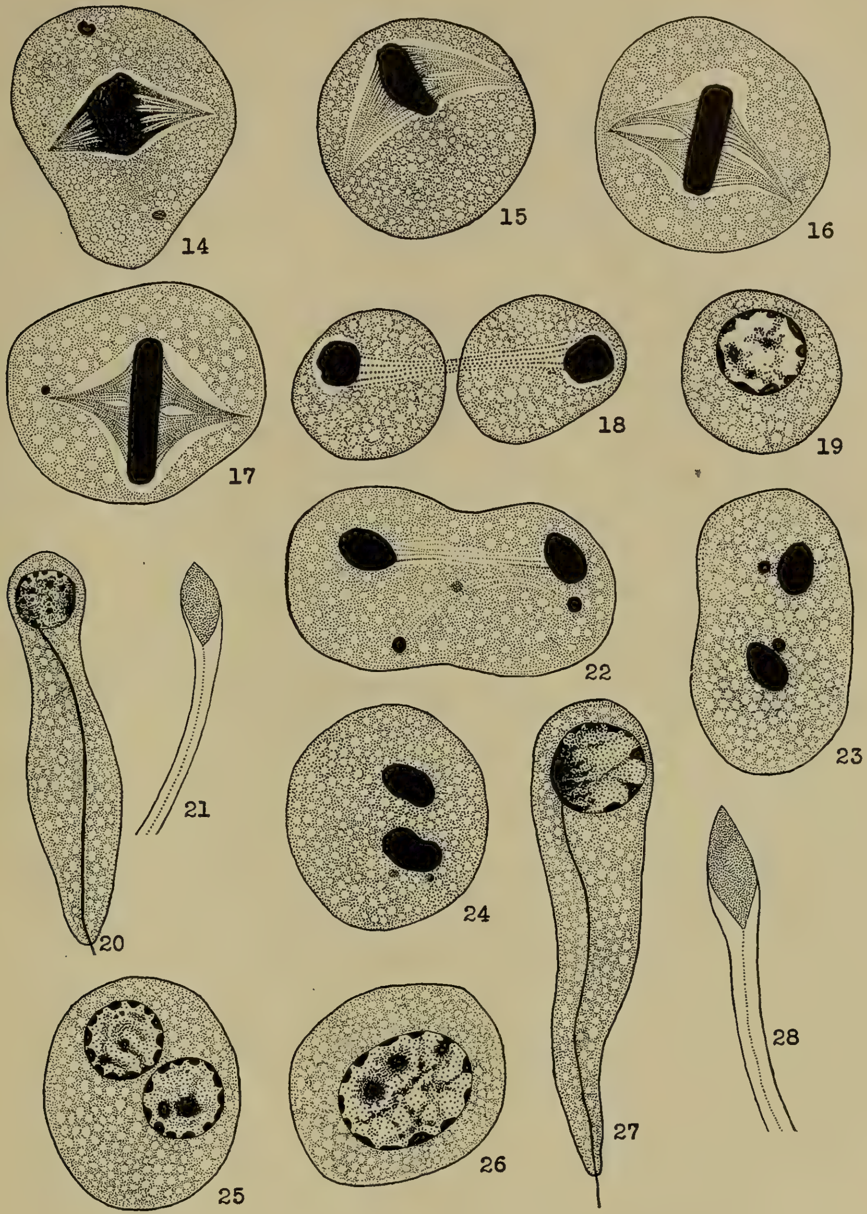
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Plate II

(1935) reports half spindle formation in connection with the condensed group of chromosomes in the germ cells of *Phenacoccus acericola,* reduction being effected by the consequent passage of the condensed group to the single pole of the spindle. Scott (1936) found that a unipolar spindle is regularly formed in the first spermatocyte division of a haploid parthenogenetically produced beetle, *Micromalthus debilis.* The monopolar spindle described in connection with the present work is similar in its general features to those already reported. They are all identified with the maturation divisions. However, the spindles reported by Metz and Sally Hughes-Schrader are found in diploid animals. The monopolar spindle here described and the one reported by Scott are found in the first spermatocyte of haploid animals, and therefore are in particular agreement as to time and place of formation.

It is commonly assumed that spindle fibers are in some way associated with a bipolar tension. In the light of these monopolar spindles it would seem that spindle fibers may exist entirely independent of any bipolar condition.

Functional Reality of Half Spindles

Anaphasic movement of chromosomes such as that described here presents an interesting problem. It has generally been assumed that whatever the nature of the half spindle may be, it is concerned with, if not responsible for, this anaphasic movement. Quite obviously, the anaphasic movement here described in connection with the first spermatocyte is in no capacity identified with, and is entirely independent of, the half spindle fibers.

The movement of these chromosomes is no doubt something like that described by Metz (1933) for *Sciara* during the first spermatocyte segregation of chromosomes or similar to the movement reported by Scott (1936) for the chromosomes during the anaphase of the first spermatocyte division in *Micromalthus debilis.* Metz indicates that the movement in *Sciara* is an " autonomous " movement on the part of the chromosomes, and that the half spindle fibers could exert only a retarding influence.

In view of these cases where half spindles play no part in the anaphase movement of chromosomes, it is quite obvious that any theory which attempts to attribute the force responsible for the chromosomal movement to half spindles or their components is not correct in its entirety.

Morphological Reality and Origin of Half Spindle Fibers

Some evidence exists for the morphological reality of half spindle fibers. Perhaps the most convincing evidence, as pointed out by Schrader (1934), pivots on the simple argument that if the half spindle or their components can be made to bend, it is evidence for their morphological reality. Schrader noted that metaphase half spindles or their components undergo bending and distortion in centrifuged cells, and in view of this experimentally produced evidence concluded that the half spindle components have morphological reality.

If bending and distortion of half spindles or their component fibers is evidence for their morphological identity, then perhaps the evidence afforded by the present study is most convincing. In the first place extreme bending of fibers is observed when the telophase chromosomes gather to form the interkinetic mass (Figs. 11–12). The chromosomes preceding drag their attached fibers with them so that in early interkinesis they radiate from the chromosomal interphase mass. In the second place the bipolar half spindles of the second spermatocyte division, in becoming oriented so that the two halves are opposed in the same plane, exhibit considerable bending (Figs. 14–16) as evidenced in the crescentic figures. The phenomena are even more convincing when it is realized that they are natural phenomena and not artificially produced.

Perhaps further evidence for the morphological reality of half spindle fibers is forthcoming in view of the apparent manner in which the spindle components arise. Carothers (1934) has especially emphasized that half spindle fibers are of chromosomal origin. In the present study it appears that the monopolar spindle of the first spermatocyte and the bipolar spindle of the second spermatocyte arise as outgrowths of some substance contained within the chromosomes. If the spindle fibers are outgrowths of the chromosomes, this would appear to be evidence *per se* that they do have morphological reality.

SUMMARY

The number of chromosomes as demonstrated by the cytological study of gonial cells is thirteen in the male and twenty-six in the female. Experimentally it was found that virgin females gave rise only to males. In view of the experimental and cytological evidence it is concluded that males having the haploid chromosome number develop from unfertilized eggs and are thus produced parthenogenetically.

The pinching off of a non-nucleated cytoplasmic bud characterizes the first meiotic division.

A monopolar spindle is regularly formed simultaneously with the pinching off of the first spermatocyte bud. This spindle would appear to be of chromatin origin.

The chromosomes or chromosomal masses, with spindle fibers trailing, exhibit pronounced anaphasic movement identified with the first spermatocyte division. The force responsible for this anaphasic movement apparently is entirely independent of the half spindle fibers.

There is no formation of a nuclear membrane between the first and second spermatocyte divisions. However, after their characteristic anaphasic movement the chromosomes gather into a compact mass for an abbreviated interkinesis.

The tendency for the half spindle components to be dragged about by the chromosomes during the anaphase of the first spermatocyte division and during the movement of the chromosomes in the formation of the interkinetic mass would seem to indicate that half spindle fibers have morphological reality.

The bipolar spindle identified with the second spermatocyte division appears to originate as an outgrowth from the interkinetic chromosomal mass. This would seem to be evidence for morphological reality of the fibers.

The second spermatocyte division may be equal, producing two normal spermatids.

Not infrequently aberrant spermatids are formed as a result of the secondary fusing of the parts of a partially divided second spermatocyte. They have a single large nucleus which is formed as a result of the fusion of two normal nuclei.

In early spermiogenesis the development of the two types of spermatids appear to be similar except for differences in size.

At present no evidence is available to indicate that the aberrant spermatids develop into functional spermatozoa.

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BIBLIOGRAPHY

Carothers, E. E., 1934. The chromosomal origin of spindle fibers. *Anat. Rec.*, **60**: 81 (Supplement) (Abstract).

Hughes-Schrader, S., 1935. The chromosome cycle of Phenacoccus (Coccidæ). *Biol. Bull.*, **69**: 462.

Metz, C. W., 1933. Monocentric mitosis with segregation of chromosomes in Sciara and its bearing on the mechanism of mitosis. *Biol. Bull.*, **64**: 333.

MEVES, F., 1907. Die spermatocytenteilungen bei der Honigbiene (Apis mellifica L.), nebst bemerkungen über Chromatinreduktion. *Arch. f. mikr. Anat. und Entwicklungsgeschichte,* **70**: 414.

SANDERSON, ANN R., 1933. The cytology of parthenogenesis in Tenthredinidæ. *St. Andrews Univ. Publ.,* No. 33: 321.

SCHRADER, F., 1934. On the reality of spindle fibers. *Biol. Bull.,* **67**: 519.

SCHRADER, F., AND SALLY HUGHES-SCHRADER, 1931. Haploidy in Metazoa. *Quart. Rev. Biol.,* **6**: 411.

SCOTT, A. C., 1936. Haploidy and aberrant spermatogenesis in a coleopteran, Micromalthus debilis LeConte. *Wistar Institute Bibl. Ser.,* No. 277: 13.

TORVIK-GREB, M., 1935. The chromosomes of Habrobracon. *Biol. Bull.,* **68**: 25.

VANDEL, A., 1931. La Parthénogenèse. G. Doin et Cie, Paris.

PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

June 30, 1936

The effect of methylene blue on the spectrophotometric picture of hemoglobin, CO-hemoglobin and CN-hemoglobin. Matilda M. Brooks.

The purpose of these experiments was to find out the effect of injections of methylene blue on the spectrophotometric picture of hemoglobin (hb) and of CO-hb and CN-hb in rabbits. The blood was taken by heart puncture, diluted to 1 per cent with .4 per cent NH_4OH and then analyzed immediately in the spectrophotometer. The extinction coefficient at wave lengths 540/560 $m\mu = R$ were determined. These values indicate the percentage of oxy-hb present.

There were three main results found; the absorption maximum of the CN-hb curve and the curve for the blood containing methylene blue were identical with that for oxy-hb; the time curve for blood containing CO and methylene blue showed 100 per cent oxy-hb within a short time. There was no difference in the absorption of oxy-hb and CN-hb in the region of the u.v. spectrum but the absorption was greater in the case of oxy-hb than of CN-Hb when wave lengths from 7300 to 10,300 Å were used. Eggert, using the infra-red region, found that blood containing methylene blue gave the same spectrum as oxy-hb while that for blood subjected to CO even after 45 minutes aeration, gave the spectrum of CO-hb.

The conclusions are as follows: the evidence for the change of CO-hb to oxy-hb by the action of methylene blue, *without invoking the methemoglobin formation theory* seems unequivocal. This may be caused by a catalytic action on the part of methylene blue, or it may be due to a poising effect on the oxidation-reduction potential of the system making it compatible with the re-formation of oxy-hb.

The phosphatase content of the developing chick embryo. Harry J. Lipman.

The phosphatase activity of the white, of the yolk and of the embryo were determined in eggs of white Leghorn hens. The determinations were made in intervals of three days during the process of incubation. For the determinations the Brigg's modification of the Bell Doisy colorimetric method for the determination of inorganic phosphorus was used. In the technique the amount of inorganic phosphate formed by the enzyme activity is measured and calculated in total milligrams (absolute values) and in milligrams per gram of wet body weight (relative values).

The absolute values and the relative values for the whole egg at zero days incubation are 0.487 mg. phosphorus and 0.082 mg. phosphorus respectively. Both of these values rise to two maxima, one at six days and the other at fifteen days of incubation, and in doing so run in a parallel fashion. The relative phosphatase values for white, yolk and embryo and the absolute values for white and yolk show these same two maxima. However, the absolute data for the embryo give a fairly good S-type curve. The steepest part of the curve coincides with the period of high ossification activity in the embryo. When the absolute and relative values for the embryo are plotted together on the same graph, the drop of the relative values after the fifteenth day stands out conspicuously against the continuous S-type rise of the absolute values. This drop can be interpreted by the fact that from this day on the growth of the embryo proceeds at a very rapid rate with which the increase in phosphatase activity does not hold pace. It was found that the material used had a high coefficient of variation. This was also true for the experimental data. These facts make the results less significant than was expected.

The description of an oxidative process maintaining the frequency of the heart beat. Kenneth C. Fisher and Laurence Irving.

Oxidation-reduction potentials and potentiometric determination of ascorbic acid. Eric G. Ball.

JULY 7

Nerve cells without central processes in the fourth spinal ganglion of the frog. Alfred M. Lucas.

Serial sections of the fourth spinal nerve of the bullfrog which included the roots, spinal ganglion, dorsal rami, spinal nerve trunk, ventral ramus, communicating ramus, sympathetic trunk anterior to the fourth nerve and the celiac nerve which were stained in toto with osmic and with silver methods, furnished fiber counts in the regions mentioned. The number of myelinated fibers found in the two roots was 667 and about 259 of these passed to the dorsal rami leaving 408 fibers for the spinal nerve, but distal to the ganglion 950 myelinated fibers were counted. Total fiber counts from silver preparations revealed 1,372 in the dorsal root, 648 in the ventral root and 883 subtracted passing to the dorsal ramus left 1,137 for the spinal nerve. Actual count of the spinal nerve showed 5,277 fibers. The additional fibers arose from the dorsal root ganglion in which were located 5,220 cells. It is concluded, therefore, that some 3,500 cells are present in the spinal ganglion which lack central processes passing through the dorsal root to the cord. The distal processes pass through the communicating ramus to the celiac nerve. Other interpretations have been considered, namely, that dorsal root fibers might have branched in the ganglion, that during development the sympathetic ganglion failed to migrate peripherally as expected, and that the missing central processes had been overlooked when counts were made on the dorsal root.

Receptor areas in the venæ cavæ and the pulmonary veins and their relation to Bainbridge's reflex. José F. Nonidez.

Extinction of reflex responses in the rat. C. Ladd Prosser.

Factors influencing the electrical activity of the brain. R. W. Gerard.

JULY 14

The permeability of the erythrocytes of the ground-hog. A. K. Parpart.

The red cells of the ground-hog have been found to be very much more permeable to a variety of dissolved substances than those of any other species thus far studied. The penetration of non-hemolytic non-electrolytes from isosmotic solutions was followed by the hemolysis method, using photoelectric recording. A few typical results are given below, the figures indicating the time in seconds required for 75 per cent hemolysis in 0.3 M solutions at 20° C.: ethylene glycol, 1.9; glycerol, 2.2; diethylene glycol, 3.0; triethylene glycol, 5.3; erythritol, 5.8; sorbitol, 59.0; mannitol, 80.0; d xylose, 133; d-l arabinose, 256; glucose, 1080; propyl alcohol, 1.4; α, β dioxypropane, 1.9; α, γ dioxypropane, 4.5; water, 0.9. The permeability of these cells to glycerol is only slightly reduced by $NaHCO_3$, Cu^{++}, and CO_2. The red cells of the ground-hog combine the permeability characteristics of those of man and the mouse. Those of man are known to be permeable to sugars but not to polyhydric alcohols such as mannitol, the reverse is true in the mouse, while the ground-hog erythrocytes are readily permeable to both of these types of substances.

Effects of salts on the injury potentials of frog's muscle. H. Burr Steinbach.

Variations in injury potential were noted as the salt content of the fluid bathing the uninjured portion of the frog sartorius was varied between the limits 0.1 molar to 0.0005 molar. When the observed potential differences are plotted against logarithms of concentration of salt applied, regular curves result. The shapes of these curves can be predicted by the equations for diffusion potentials for such salts as NaCl and $NaC_2H_3O_2$. With high concentrations bathing the uninjured surface the slope of the curve is characteristic of the salt applied. With low concentrations the slope of the curve is determined by the electrolytes of the tissue and is nearly independent of the nature of the salt in the solution. With KCl solutions, the p.d/ log concentration curve is as calculated for concentrations less than 0.01 molar. With concentrations 0.01 molar or greater, the observed potentials become increasingly negative to the calculated as the KCl concentration increases. With isotonic (ca 0.1 molar) KCl, the difference between observed and calculated values is 40 to 50 millivolts, or nearly equal to the injury potential itself. A value of 40 to 50 millivolts must be added to calculated values in order to obtain a quantitative agreement with the observed values for NaCl, $NaC_2H_3O_2$ and the dilute range of KCl. It is concluded that the presence or concentration of such indifferent salts as NaCl and $NaC_2H_3O_2$ (when they are applied to the uninjured surface) has little effect on the injury potential. Potassium, on the other hand, in concentrations 0.01 molar or greater, acts on the uninjured surface to lower the measured injury potential, probably by producing "injury" at the point of application. The concentration at which KCl first shows specific effects is about that at which KCl contracture sets in.

Free calcium in the action of stimulating agents on Elodea cells. Daniel Mazia and Jean M. Clark. (The full paper appears in this issue of the BIOLOGICAL BULLETIN.)

A photoelectric method for recording past chemical reactions. Application to the study of catalyst-substrate compounds. Kurt G. Stern and Delafield DuBois.

JULY 21

Observations on lens regeneration in Amblystoma. W. W. Ballard.

In the literature, *Amblystoma mexicanum* is said to be capable of Wolffian lens regeneration at all stages in its life history. It is found that *Amblystoma punctatum* loses this ability very rapidly in late embryonic stages. At stages 38–39, lenses are formed and separated from the iris in 4 days in 100 per cent of cases. At stage 43, six days later, practically no lens regeneration is obtained. Similar results were gotten from embryos of *A. tigrinum*, and *A. microstomum*. During larval stages of *A. punctatum*, *A. tigrinum*, *A. microstomum*, *A. jeffersonianum* and *A. opacum*, no lens regeneration was obtained over periods up to four months after extirpation.

Using uniform material, a greater percentage of *A. punctatum* embryos regenerated lenses in light than in darkness, at 5° C. than at room temperature, in 6/8 Ringer's solution than in 1/8 Ringer's.

Parabiotic twins were formed in *Amblystoma punctatum* at stages 38–41 in which one lens lay in the pupil between two eyeballs whose irises were superimposed.

Of these two eyeballs, the one which had previously been deprived of its lens regenerated a new one, in spite of the presence partly within it of the lens of the other eye.

Fertile eggs from pheasants in January by night-lighting. T. H. Bissonnette.

A quantitative analysis of the anterior pituitary-ovulation relation in the frog. Roberts Rugh.

Since the original work by Dr. O. Wolf in 1929, ovulation has been induced in an increasing variety of amphibia. In 1934 it was first suggested that while either male or female glands could be used to induce ovulation, there was a potency difference favoring the female anterior pituitary. Since the response to anterior pituitary injection was not uniformly dependable, it seemed desirable to study this relationship quantitatively.

The work was divided into two phases: first, an attempt was made to determine any correlations between sex, body weight, body length, gonad weight, and anterior pituitary weight. The second phase was a study of the degree of ovulation elicited by quantitatively determined doses of the gland.

Over 700 frogs were used, covering two periods: November and February. These months were chosen as representing the beginning and the end of the normal hibernation period. Since it was found that anterior pituitaries varied in weight from 0.6–1.6 mgm., in order to lessen observational error the glands were removed from frogs of approximately the same body length and were weighed in groups of twenty.

While the body lengths of males and females averaged almost the same for November and February, there was consistent reduction in average body weight and average gonad weight, over this hibernation period. Body length, as measured from nares to cloacal opening, was the standard by which other variables were considered. It was found that males and females with body lengths less than 71 mm. were immature; that those between 71–74 mm. showed the beginning of sex differentiation and gonad growth; and males and females longer than 74 mm. showed sex differences in the relative weights of their anterior pituitaries. These differences favored the males, both in November and in February. Over this hibernation period there was considerable reduction in relative weight of the anterior pituitaries in both sexes, at some stages this was as much as 29 per cent. There is a similar reduction, over the hibernation period, of relative ovarian weights. If anterior pituitary weights are plotted against ovarian weights, it is demonstrated that there are correlating reductions in the weights of these two organs, all points falling on a straight line for both November and for February.

In respect to the induction of ovulation, it was found that in November 8 mgm. of male anterior pituitary induced about 42 per cent of the eggs to leave the ovaries while 5 mgm. of female gland tissue induced 85 per cent ovulation. In February 4 mgm. of either male or female gland tissue would induce 100 per cent ovulation, and in doses less than this there were indicated differences in potency favoring the female gland. These seasonal differences may be due to hibernation concentration of the donor's anterior pituitary; to greater susceptibility of ovaries in February than in November to the anterior pituitary hormone; or even to the activation in this prebreeding period of the recipient's own anterior pituitary.

The anterior pituitaries removed from females which had been induced to ovulate showed no decrease in potency in respect to inducing ovulation in other females. This supports the thesis that the injection of the hormone is comparable to the liberation of the host's hormone, and that the host's hormone is in no way affected.

If frogs are selected at random, it will be found that the average male anterior pituitary is about 16 per cent heavier than that of the average female, but is only

60 per cent as potent in respect to inducing ovulation. This supports the thesis that the female glands are approximately twice as potent as the male glands.

Like many biological processes, which at first seem to be simple, this relationship between the anterior pituitary and ovulation resolves itself into a number of variables: (1) size and sexual maturity of the donor of the anterior pituitary; (2) concentration of the hormone through hibernation reduction in weight; (3) activity of the recipient's own gland; (4) source of the hormone, i.e., from male or from female; (5) dose of the hormone (mgm. of gland tissue); (6) size and sexual maturity of the recipient; and (7) size and susceptibility (maturity) of the ovaries to ovulation induction.

This quantitative study points toward seasonal as well as metamorphic changes in the anterior pituitary which must be studied from the cytological point of view. This study is at present being made on the bullfrog, *Rana catesbiana*, and will be reported subsequently. (This paper will appear in full in the January, 1937 issue of *Physiological Zoology*.)

Experimentally induced coupling in the toad, Xenopus lævis. (Moving picture.) H. A. Shapiro.

July 28

The relation between vitamins and the growth and survival of goldfishes in homotypically conditioned water. Gertrude Evans.

A possible endocrine rôle of the eosinophil leucocytes in the female rat. C. P. Kraatz. (The full paper will appear shortly in the *American Journal of Physiology*.)

An interpretation of the secondary lymphoid nodules in the albino rat. J. E. Kindred.

Quantitative data from a detailed study of serial sections of submaxillary, inguinal and mesenteric lymph nodes, and Peyer's patches of eight 80-day old albino rats from the same litter, are presented in support of Röhlich's view that the secondary lymphoid nodule is both a germinal center and a center of reaction to substances in the blood stream.

The secondary nodules are prolate spheroidal or spherical in shape and each is roughly divided into a light and a dark zone which differ quantitatively from each other in respect to rates of mitosis, incidences of macrophages, and the several varieties of lymphocytes. The light zones are capped externally by a cup-shaped layer of small lymphocytes and reticulum. The blood capillary plexus of the nodule lies in the light zone.

Because the dark zone has a statistically significant higher rate of mitosis than the light zone in all nodules studied (e.g. mitosis per 1,000 cells with standard error of mean, submaxillary nodes: dark zone 31 (2.3); light zone, 6.3 (0.3)), it is concluded that the dark zone is the germinal center of the nodule. On the other hand, the presence of a blood capillary plexus, approximately equal incidences of small lymphocytes (e.g. 54 per cent (1.6) in mesenteric nodes; 60 per cent (0.6) in Peyer's patches) and macrophages containing degenerating nuclei of small lymphocytes (e.g. 52 (6.3) per 1,000 cells in mesenteric nodes; and 42 (2.3) in Peyer's patches) in the light zones of the nodules of both lymph nodes and Peyer's patches, suggests that the functional activity of the light zone is related to the local reaction of small lymphocytes to substances in the blood. The quantitative data suggest that the minimum mitosis rate necessary to maintain this relation is the rate in the dark zones of Peyer's patches (e.g. 15.5 (0.5) per 1,000 cells). The close relation between the nodules of the lymph nodes and the lymphatic channels, together with the higher rates of mitosis in the dark zones (e.g. submaxillary nodes, 31 (2.3); inguinals, 32 (2.4); mesenterics, 30 (2.5) per 1,000 cells) suggest that not only do the dark zones of the nodules of the lymph nodes function to maintain a local balance of small lymphocytes, but in addition contribute small lymphocytes to the lymph stream.

From the fact that medium-sized lymphocytes predominate in the dark zones (e.g. submaxillary nodes; dark zone 80 per cent; light zone, 40 per cent) and are the cells most frequently found in mitosis (e.g. submaxillary nodes; 90 per cent of cells in mitosis are medium-sized lymphocytes), it is concluded that the medium-sized lymphocytes are the lymphoblasts.

Physiological adjustments to diving in the beaver. Laurence Irving.

When a normal beaver ceases breathing during submersion, its heart is inhibited. During periodic breathing, anesthetized beaver showed slowing of the heart and a greater than fifty per cent fall in arterial blood pressure during the intervals between breathing movements. A similar fall in blood pressure, slowing of the heart and inhibition of breathing movements followed from gentle inflation of the lungs of the beaver. In addition it was observed that the blood flow through the hind leg was nearly arrested when the lungs were inflated. It was not obvious to what extent constriction of the vessels in the leg contributed to the retarded blood flow, but other observations indicated that the peripheral blood vessels were able to change blood flow rhythmically. Apparently the depressor effect of inflation of the lungs is the same in the beaver as it is in other animals. The difference in the beaver is in the accentuation of the response, which easily arrests breathing for a minute, slows the frequency and decreases the force of the heart and very nearly arrests blood flow through the muscles. These responses are part of the respiratory adjustment of diving animals for enduring asphyxia. It is suggested that the stimulus of inflation of the lungs occurs at the start of a dive and brings about this sequence of physiological adjustments which prepare for asphyxial conditions before the chemical stimuli are effective. The responses seen in the beaver following inflation of the lungs are like those of other mammals except that they are quantitatively greater.

AUGUST 4

Development of Arbacia eggs without nuclei: parthenogenetic merogony. Ethel Browne Harvey. (The full article appeared in the August, 1936 issue of the BIOLOGICAL BULLETIN, vol. 71, pp. 101–121.)

Cortical changes in Arbacia eggs during fertilization—a moving picture. F. Moser.

Temperature effects on mitotic changes in Arbacia eggs. H. J. Fry.

Sea-urchin larvae with cytoplasm of one species and nucleus of another. Sven Hörstadius. (The full report has already appeared in *Mem. Mus. d'Hist. nat., Bruxelles*, Ser. 2, vol. 3.)

AUGUST 11

A study of the cells of the adrenal gland of the ewe during estrus and pregnancy. Laura J. Nahm.

Studies in calcification: III. The shell of the hen's egg. E. Alfred Wolf and Grace Riethmiller.

In two previous papers by McBride and Wolf it was shown that the Gomori modification of the silver nitrate method can be successfully applied to the study of early stages of calcification of bone and teeth. Since these structures are composed mainly of calcium phosphate, it was desirable to test the method on calcified structures that are predominantly carbonates; the shell of hen's egg was selected in which 89–97 per cent is calcium carbonate and only 0.5–5 per cent calcium phosphate.

The eggshell shows three layers: a thin, hard, structureless outer membrane, the main calcareous body and an inner fibrous layer into which dip the calcified nipples of the main body. After treatment the shells were dark on both surfaces showing where the silver nitrate had reacted, but the main body of the shell was still white and hard; decalcification therefore was required. Cross sections through the main part of the shell showed no traces of silver, except for a brown color of the surface membrane. The fibrous membrane under low power magnification had the appearance of a leopard skin: a large number of black spots were more or less evenly scattered over the area of the membrane, the fibers of which were light brown in color. Under high magnification (oil) it could be seen that the spots were due to a precipitation of silver within the fibers themselves. This is taken as an indication of the location of the calcium phosphate deposits in the eggshell.

The failure of the calcium carbonate to react in the case of the eggshell would then suggest that our description of the process of calcium deposition in bone and teeth of rat embryos, as it was given last year, was incomplete since it did not include the carbonate portion (6 per cent) of the calcifying structures.

Diffraction patterns of striated muscle and sarcomere behavior during contraction. Alexander Sandow.

A method has been devised that permits photographically recording the diffraction patterns formed by 1 mm. long segments (i.e., about 400 striations) at different positions along the length of the frog sartorius (*Rana pipiens*) over the course of single contractions. The simultaneously produced myograms are included on the records.

The patterns produced by a set of different segments of the sartorius undergoing isometric twitch, supermaximally stimulated at *in situ* length, include the following: all records have 1st order spectra of practically unchanged intensity over the whole course of the twitch; the displacements of the 1st order spectra produced by the segments of the tibial two-thirds of the muscle indicate that, in relation to resting length, the corresponding sarcomeres are shortening, while the patterns of the pelvic-third segments show that here the sarcomeres are being stretched. The nature of the shortening and stretching of the sarcomeres at the various muscle levels indicates that the contraction wave is initiated at the nerve plexus and then spreads up and down the muscle away from this region.

During supermaximally stimulated moderately loaded isotonic contractions the patterns show 1st order spectra similarly unchanged in intensity, but with very great displacements, indicating sarcomere shortening up to 20 per cent; contraction, however, takes place at all levels of the muscle.

During both isometric and isotonic contractions the 0th order increases, and the 2nd order decreases in intensity.

The above results, particularly the intensity variations, are interpreted to mean that Jordan's description of contracting sarcomere behavior (*Physiol. Rev.*, **13**: 301, 1933) does not hold for the frog sartorius; but that in the contracting frog sartorius: (1) a sarcomere, in the sense of consisting of a Q band flanked at each end by one-half of each of the adjoining I bands, remains intact, although (2) it may contract or be stretched depending on its position in the muscle and the nature of the contraction; and (3) there occurs an unequal change in the refractive indices of the Q and I substances, or a variation in the ratio of the lengths of Q and I bands, or both of these modifications. And whatever the directions of these changes for contraction of sarcomeres, they are probably opposite in direction for stretch.

The effect of some oxidation-reduction indicator dyes (phenol indophenol) on the eyes and pigmentation of normal and hypophysectomized amphibians. Frank H. J. Figge.

The discovery of the Lewises that the indophenol dyes produced a marked pallor of the skin and loss of the pigmented layer of the optic cup in tadpoles was used to study amphibian pigmentation. Phenol indophenol and ortho-cresol indophenol were tested on a series of six kinds of amphibian eggs: normal and hypophysectomized *Amblystoma punctatum*, black and white Mexican axolotl, Necturi, and bullfrog. The hypophysectomies were performed between the Harrison stages 30–37. The animals were placed in the dye solutions (1 : 500,000 and 1 : 1,000,000) near the hatching stages and kept there continuously for a period of three months. As the animals grew, it was necessary to increase the concentrations. Dye solutions were changed daily, and during the last half of the experiment, twice daily.

From a study of the relative sensitivity of the salamanders to the dye, it was evident that the degree of sensitivity paralleled the metabolic rate. The higher the metabolic rate, the less sensitive the animal, and less effective the dye. The dyes eliminated the pigment from the tails of hypophysectomized *Amblystoma punctatum* larvae, proving conclusively that the dyes do not exert their influence on the melanophores through any intermediate effect on the hypophysis or its products. The white axolotls decolorized the dye as rapidly as the black. The black animals became as white as the white axolotls. The rate of decolorization of the dye was, therefore, not related to the rate of pigment destruction. The dyes were most effective in eliminating the pigment from *Necturus*, the form closely resembling cave salamanders that exhibit pigment and eye defects similar to those produced by the dyes.

The animals raised in dye solutions exhibit eye defects that vary in degree and uniformity with the species, within the species, and even in the same individual. These defects involve not only the retina and pigmented layer of the eye, but also the scleral cartilage, lens and cornea.

The elimination of neutral red by the kidney tubules. Robert Chambers.

AUGUST 18

Quantitative studies on blood clotting. H. P. Smith and E. D. Warner.

The basis of the principle of the master reaction in biology. Alan C. Burton.

The principle that the rate of a chain of processes is determined dominantly by the speed of the slowest process in the chain underlies the widely used method of "temperature analysis" of complex biological processes. Its origin in the analysis of a chain of two irreversible monomolecular reactions is traced. A table is calculated showing how much one velocity constant must be slower than the other in this system in order that the slower reaction is within a given percentage of complete dominance of the chain. This requirement is so exacting that when the Arrhenius law of rate of change with temperature is followed, it is impossible that one reaction may be within 10 per cent of a true master reaction at 0° C. and the other similarly dominant at 40° C. unless the "critical increments," μ, differ by at least 16,500 Calories. Smaller temperature ranges require proportionately greater differences in the critical increments. The straightness of a line through observed points on an Arrhenius plot may result from dominance shared by a number of reactions rather than of a single master reaction.

In longer, more complicated chains the requirements for a true master reaction are more exacting, and for systems in the "steady state" the principle has no application.

It is concluded that the principle of the master reaction has a very restricted application even in these idealised chains and still less to the real systems of biology.

Efficiency of photosynthesis in purple bacteria. C. S. French.

Streptococcus varians assimilates carbon dioxide with hydrogen in the light but not in the dark. The effect of hydrogen pressure, pH, wave length, intensity, and temperature on the rate of the reaction is described. The curves of rate against intensity are S-shaped and influenced by the previous treatment of the bacteria.

Using light of λ 852 with λ 894 from a caesium tube the quantum yield of a thin suspension was measured. About four quanta are needed per carbon dioxide molecule reduced, thus pointing to a similarity with green plant photosynthesis in spite of the fact that the net result of the assimilation does not involve an increase of bound energy.

Some effects of ultraviolet radiation on bacteria. Alexander Hollaender. (Much of this material has already been published—*see Jour. Gen. Physiol.*, May 20, 1936 and *Proc. Soc. Exper. Biol. and Med.*, 1935, vol. 33, and a part of the data will be published in collaboration with Dr. Duggar shortly. A monograph containing some phases of the work is in print.)

Myelination in the central nervous system of the albino rat, treated with thymus extract. (Microscopic demonstration.) Albert C. Buckley.

AUGUST 25

Cellular behavior in abnormal growths produced by irradiation of grasshopper embryos. E. Eleanor Carothers.

Embryos of *Melanoplus differentialis*, ranging in age from five to nine days, received either 250 or 300*r* units in a single exposure to X-rays. The treated eggs and controls from the same lot were kept at 25° C. and embryos from each group were fixed daily until the eggs were well into diapause. Roughly 50 per cent of the embryos from the treated eggs show abnormal growths which are duplications and misplacements of normal parts. The majority of these structures occur in the head region. Sections of embryos 24 hours after irradiation show cells with pycnotic nuclei scattered in groups throughout the germ band. These pycnotic nuclei are formed by the coalescing of the chromatin at the end of the anaphase to form a homogeneous, basiphilic globule. Such cells are either phagocytized by other tissue cells, absorbed in situ or cast out of the embryo. Forty-eight hours after irradiation some metaphases show fragmented and lagging chromosomes. The most striking feature at this stage is another type of degeneration. The cytoplasm cytolyzes beginning at the periphery. The spongioplasm remains resistant for a time. The nuclear membrane disappears but the chromatin remains normal in appearance. Gradually it too goes into solution without clumping. By the fifth day after irradiation cytolytic lesions have been formed. The cytolysis may be due to (1) injury to the cytoplasm by the X-rays (2) failure or destruction of some other group of cells upon which the cytolyzed cells are normally dependent (3) the phagocytizing of pycnotic cells which transmit a toxic effect. Conclusion: The abnormal growths seem to be the product of groups of normal, healthy cells which either by cytolytic or pycnotic lesions are separated from their normal association and proceed to form as much of a new embryo as their cells retain the potentiality to develop. The original pycnosis is clearly traceable to definite stages of mitosis at the time of treatment.

The behavior of the cell surface during cleavage. Katsuma Dan, T. Yanagita and M. Sugiyama.

To find out whether the increase in cell surface which must accompany cleavage occurs uniformly over the egg surface or whether it is different in different regions, particles of kaolin were stuck to the naked eggs of the sand dollar *Astriclypeus*. During subsequent segmentation the positions of these particles were recorded by camera lucida outline drawings and photomicrographs. The data obtained by this method represent linear expansion.

As elongation of the cleaving egg begins, the surface near the spindle poles expands, reaches a maximum (ca. 20 per cent increase) about halfway through cleavage, then shrinks somewhat, arriving at a stable value about 5 per cent greater than the initial reading. On the other hand, measurements taken simultaneously in the region of the cleavage furrow show first a slight shrinkage (ca. 20 per cent), then a very large increase (ca. 150 per cent) occurring later than that at the poles (at completion of cleavage furrow) and finally a shrinkage to a stable value which is again much higher than that at the poles (ca. 80 per cent increase over initial reading). The egg surface between these extremes shows intermediate behavior both in magnitude of the maximum increase and retardation of its appearance.

*Modified sexual photoperiodicity in ferrets, raccoons and quail.** T. H. Bissonnette.

In the work with raccoons (*Procyon lotor* (L.)), about fifty controls were used of both sexes, whose behavior and times of pseudo-hibernation and breeding were normal. Three groups (each of one ♂, one breeding ♀, previously successful, and one unsuccessful) were lighted by 60-watt bulbs from October 10, one hour per night for a week, increased by an hour each ten days to eight hours per night from December 10 onward. All had usual care and identical climatic conditions outdoors with temperature from above freezing to − 20° F., and deep snow occasionally. Some ♀ ♀ were removed from lighted cages when they became pugnacious toward the end of gestation or pseudo-pregnant periods; others were not; but without affecting pregnancies or suckling.

Lighted animals did not decrease food consumption nor undergo pseudo-hibernation with onset of cold weather in December and early January as did controls. All females in lighted pens mated from December 16, 19, and 23, respectively, for ten or more days; controls only after February 1 or even April 15 (38–40 days later) with first litters on April 6 and 7 as in the previous year. The three previously sterile ♀ ♀ again failed to have litters; but the productive ones gave litters of four on February 27, one on March 4, and three on March 10, respectively (exact numbers of preceding "normal" season), and suckled and weaned them through sub-zero weather. The former, returned to lighting and males on March 20, again mated without conceiving; of the latter, returned to lighting and males on May 18 and 19, after suckling, two mated from May 24 onward, the other probably did so unobserved; but no young resulted from these second matings. Second mating periods did not occur with controls nor in these ♀ ♀ in their previous "normal" season. Male and female cycles synchronized.

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* The data on ferrets will appear in the *American Naturalist* shortly. The data on quail will appear in the October number of *Bird Banding* and need not be gone into here.

Asexual reproduction in Dodecaceria fimbriatus. Earl A. Martin.

Dodecaceria fimbriatus reproduces sexually by pelagic epitokes and asexually by autotomizing single segments in the middle region of the body. This worm lives in a flask-shaped burrow in which it is folded on itself so that the dorsal surfaces are apposed. The fold is located immediately posterior to the stomach in the intestinal region. When a worm is removed from its burrow in the calcareous materials in which it lives, and covered with loose sand or powdered limestone, it folds on itself and forms a burrow similar to those in the normal habitat. This shows that the folding is a fundamental characteristic which must be considered in relation to the morphology and physiology of the worm. When considered from this point of view, the anterior limb of the fold consists of the feeding and digestive mechanisms, while the folded region and the posterior limb, in addition to conveying the faeces to the anus, serves primarily for the storage of reserve food in the cœlome. Single segments are autotomized in this part of the body.

All autotomized units of the worm regenerate. The anterior part regenerates a new posterior end and the posterior part regenerates a new anterior end. A single segment regenerates from eight to twelve segments anteriorly and about thirteen posteriorly. From this stage growth may continue until a complete worm is formed or the anterior and posterior ends may autotomize at the junction with the original segment each of these autotomized parts then forming complete worms.

The physiology of the stomatogastric system in Arenicola marina. G. P. Wells.*

A series of lugworms (*Arenicola marina*) were watched in U-tubes of sea water. The majority showed an activity cycle, periods of rhythmic head movements or of rhythmic extrusion and withdrawal of the proboscis alternating with periods of rest. The duration of a single cycle is somewhat variable, but is commonly six or seven minutes. Experiments on worms dissected in various ways show that the pacemaker determining this phenomenon is a diffuse structure, probably a nerve-net, in the wall of the œsophagus. From this structure, periodic outbursts of excitation travel forward by way of the proboscis to the central nervous system, evoking outbursts of rhythmic activity in the muscles of the proboscis and anterior body wall. Adrenaline excites the œsophageal pacemaker, the rhythmic discharge becoming continuous instead of intermittent. Injected into intact worms, adrenaline causes prolonged, continuous burrowing. The work was done in England, at Plymouth and London, and a detailed report will appear shortly.

General Scientific Meeting

August 27

The effect of lack of oxygen on the permeability of the egg of Arbacia punctulata. F. R. Hunter and E. N. Harvey.

Fertilized and unfertilized eggs were studied in the presence and absence of oxygen under the following conditions: (1) when placed in hypotonic sea water, (2) in hypertonic sea water, (3) in a 0.475 molar ethylene glycol solution in 50 per cent sea water, and (4) in a 0.5 molar ethylene glycol solution in 100 per cent sea water. Eggs which were shrunk in 200 per cent sea water under anaerobic conditions showed a peculiar cortical change. Under the other conditions studied, lack of oxygen had no effect on the permeability of these eggs. These results are in conflict with the slight effect of anaerobic conditions on permeability which had previously been reported by Keckwick and Harvey (*Jour. Cell. and Comp. Physiol.*, **5**: 43–51, 1934) and Hunter and Harvey (BIOL. BULL., **69**: 344, 1935). It is suggested that the previous conclusions were due to incorrect interpretation of the data.

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A photronic cell was used in measuring the volume changes in contrast to direct measurements of eggs as had been made during the preceding summer. In this way several hundred thousand eggs could be measured at one time, whereas in the previous experiments only a few cells were measured.

Comparative permeability to water and certain solutes of the egg cells of three marine invertebrates (Arbacia, Cumingia and Chætopterus). B. Lucké, R. Ricca and H. K. Hartline.

Permeability of the cell to water is defined as the amount of water that passes per minute through one square micron of cell surface with a difference in osmotic pressure of one atmosphere. This quantity has been determined for the egg cells of the mollusk *Cumingia tellenoides*, the annelid *Chaetopterus pergamentaceus* and the echinoderm *Arbacia punctulata* by measuring the volume changes of these cells in hypotonic sea water by means of a diffraction method.

The course of osmotic swelling of the three kinds of cells is found to be satisfactorily described by the equation

$$\frac{dV}{dt} = K \cdot S \cdot (P - P_{ex}),$$

where dV/dt is the rate of volume change in cubic micra per minute, S' the cell surface, $P - P_{ex}$ the difference in osmotic pressure between cell and medium at time, t, and K is defined as the permeability of the cell to water. For *Arbacia* eggs the values of permeability range around 0.1 at 22° C.; the eggs of *Cumingia* and of *Chætopterus* have much higher values which range from 0.4 to 0.5 cubic micra per minute, per square micron of cell surface, per atmosphere.

Permeability to the rapidly penetrating solute ethylene glycol and to the slowly penetrating solute glycerol is investigated by means of a method devised by Jacobs and Stewart. The cells are placed in a medium made hypertonic by dissolving a quantity of the penetrating substance in sea water. In this hypertonic solution the cells at first shrink, but as the solute becomes distributed between the cells and the medium they gradually return to their original size. From the course of such volume changes permeability to the solute is computed. The values are expressed as the number of mols times 10^{-15} which pass per minute through one square micron of cell surface with a concentration difference of one mol per liter.

Values of permeability to ethylene glycol are found to average approximately 4.0 for *Arbacia* eggs; for *Cumingia* and for *Chætopterus* cells the values are approximately 16.0 at 22° C. Glycerol penetrates very slowly into *Arbacia* eggs; the average value for permeability is 0.03; *Chætopterus* eggs have a much higher value which ranges around 6.0.

It is concluded that the egg cells of *Cumingia* and of *Chætopterus* are several times more permeable to water, and to ethylene glycol and glycerol than are the eggs of *Arbacia*.

Permeability of Ameba proteus to ions. S. A. Corson.

A kinetic method of studying surface forces in the egg of Arbacia. F. J. M. Sichel and A. C. Burton.

Estimates of the surface forces of cells have in the past been made by static methods. The procedure used in the present study is a kinetic one and therefore of value although perhaps less accurate.

The measurements were made from a motion picture taken by Dr. Robert Chambers for the purpose of studying the nature of cleavage in the *Arbacia* eggs from which all extraneous membranes had been removed after fertilization. The pictures were made available to us through the courtesy of Dr. Chambers to investigate the part of the problem under discussion.

If, just prior to the completion of the first cleavage, one of the blastomeres be ruptured with a microneedle, its contents are rapidly discharged into the surrounding sea water. If this be done at the proper stage of development, the contents of the remaining blastomere will be discharged through the connecting stalk. This discharge is due to an excess internal pressure which is in equilibrium with the surface forces.

The rate of discharge follows a law which can be explained by elastic forces rather than by surface tension. Assuming Poiseuille's law and Heilbrunn's value for the viscosity at the stage of development under consideration, we arrive at maximum values of 62 dynes per cm^2. for the excess internal pressure and 0.09 dynes per cm. for the tangential surface forces. These are of the same order of magnitude as the determinations of Harvey and of Cole for the unfertilized egg.

Experimental studies on the oil wetting property of the plasma membrane.

R. Chambers.

An oil droplet when applied to the surface of the protoplasm of various marine eggs in sea water either adheres to the egg, the surface of contact of the oil becoming flattened, or slips through the protoplasmic surface and occupies a position in the interior with a flattened surface of contact on the inner side of the protoplasmic surface. At least two conditions are necessary for this to occur: (1) the surface of the egg protoplasm must be freed of extraneous coatings which harden in the presence of calcium, (2) the surface of the oil on being brought into contact with the egg, must be expanding in order to be clean. The animal material used were exovates of unfertilized starfish eggs and intact sea urchin (*Arbacia*) eggs carefully shaken and rinsed in calcium-free sea water and then returned to sea water. Olive oil was used. The results obtained indicate that an increase in acidity of the medium to pH 4.0 greatly enhances the slipping of the oil into the protoplasm while an increase in alkalinity to pH 9.5 decreases the effect.

Interfacial films between oil and cytoplasm. M. J. Kopac.

Minute drops (diameter, 10 to 20 μ) of percomorph liver oil injected into immature oöcytes of *Asterias* remain perfectly spherical. This condition holds for any animal, vegetal or mineral oil or non-toxic water-immiscible liquid. The injected oil drop may be readily deformed by compressing or stretching the cell, and on releasing the pressure quickly returns to a spherical shape. Upon cytolysis of the oöcyte, the oil drop becomes heavily wrinkled and considerably deformed. This wrinkling occurs in oöcytes immersed in acidified sea water (pH = 4), in sea water, or in isotonic $CaCl_2$ alkalinized to pH > 9.5. In isotonic $CaCl_2$ acidified to pH < 3.5, wrinkling occurs but never heavily enough to produce a violent deformation of the drop. The wrinkles on the surface and the collapse of the oil drop are due to the formation of a plastic solid film at the interface (cf. *inter alia*, Wilson and Fries. *Coll. Symp. Monog.*, **1**: 145 (1923)). In order to get wrinkling and deformation of the oil drop, the oöcyte must be cytolyzed within 2 minutes after the oil is injected. If an oil drop is kept inside an intact oöcyte for 5 to 10 minutes, no wrinkling or deformation will occur after the cell is cytolyzed. Apparently the surface of the oil has changed sufficiently to prevent the formation of a plastic solid film after exposure to the cytolytic products of the cell. The wrinkled oil drop may be readily impaled with a microneedle and removed from the cytolyzed area. The drop behaves as a plastic solid and may be cut into several fragments. Some of these fragments remain irregular while others become spherical as in ordinary oil drops. This behavior indicates that the interfacial film is a plastic solid which remains in place and does not affect the interior of the oil drop.

The question of recovery from X-ray effects in Arbacia sperm. P. S. Henshaw.

In our early experiments it was found that when mature *Arbacia punctulata* eggs are exposed to X-rays before fertilization the onset of cleavage following fertilization is delayed, the amount of delay varying with two known factors. The first of these is the quantity of radiation administered and the second is the amount of time allowed between treatment and the moment of insemination. The irradiation effect (i.e. cleavage delay) is increased as the amount of radiation applied is increased and reduced as the time between treatment and insemination is increased. The reduction or loss of effect may be regarded as a form of recovery, that is, as observed by determining cleavage time. The recovery process, whatever its nature, is in progress as soon as any irradiation effect is produced (even while treatment is going on), and the rate at which it takes place following treatment is practically exponential with time.

More recently it has been shown that cleavage delay may be produced by irradiating sperm alone and that the effect is to some extent accumulative when both gametes are treated. In practically all cases, however, the effect for equal exposures was found to be greater on the sperm than on the eggs.

During the present season, a series of preliminary experiments have been carried out to determine whether recovery takes place in sperm the same as in eggs, but without exception none has been observed. Thus, since the sperm cell is composed largely of nuclear material, these experiments suggest that the X-ray effect was produced mainly in the nucleus and that the cytoplasm is involved in the recovery process. Lack of recovery in the sperm will probably account for its greater susceptibility.

The respiratory effects exerted by certain organic compounds in relation to their molecular structure. Anna K. Keltch, G. H. A. Clowes and M. E. Krahl.

Previous work by the present authors has shown that nitro and dinitrophenols can, in extremely low concentrations, produce a large increase in oxygen consumption and a complete and reversible block to the division of marine eggs. Phenols containing nitroso or amino groups in place of the nitro lacked these effects. Nitro and dinitrobenzene derivatives having the OH replaced by NH_2, Cl, Br, I, CH_2OH, NR_2, OR, COOH, or $CONH_2$ also lacked these effects. From such experiments it was tentatively concluded that the effects of the nitrophenols on cell respiration and cell division were not due to a reversible oxidation or reduction of the nitro groups.

The present experiments provide an extension and confirmation of this view. Phenols containing two or three halogen substituents in the benzene ring produced respiratory stimulation and division block in fertilized *Arbacia* eggs in the same way and to approximately the same degree as paranitrophenol, dinitrocresol and 2, 4 dinitrophenol. Phenols having only one halogen substituent and no other groups were inactive.

The isomeric hydroxy benzoic acids, the isomeric hydroxy benzaldehydes, and phenols having one aldehyde and one halogen substituent were inactive.

To produce a substantial respiratory stimulation and an accompanying reversible division block in marine eggs it seems necessary to have a phenol hydroxyl group, and a suitable number and arrangement of nitro groups or halogen groups or both. Even replacement of the H of the hydroxyl group by either CH_3 or C_2H_5 rendered the phenolic compounds inactive.

Action of metabolic stimulants and depressants on cell division at varying carbon dioxide tensions. M. E. Krahl, G. H. A. Clowes and J. F. Taylor.

From previous experiments it was not known whether the division block produced in marine eggs by dinitrophenols was attributable to the phenols themselves or to secondary effects of metabolic intermediates or end products, such as an excessive amount of lactic acid produced aerobically.

The following new evidence demonstrates that abnormally large amounts of acidic metabolic products cannot be responsible for the adverse division effects of phenol derivatives: (1) Phenosafranine, an inhibitor of the Pasteur reaction, produced no inhibition of cell division. (2) Penetration of high concentrations of butyric acid produced no division block comparable to that with the phenols. Likewise, concentrations of CO_2 up to 2 per cent produced no marked inhibition of cell division. When either of these penetrating acids was employed in sublethal doses inhibition of division appeared to be equally intense at all points in the mitotic cycle whereas the nitrophenols were more effective before onset of prophase. (3) Division blocking concentrations of the phenol derivatives did not produce an abnormally high aerobic glycolysis in *Arbacia* eggs.

The division block produced in *Arbacia* eggs by a fixed concentration of dinitrocresol was dependent on egg concentration, a greater blocking being produced as the number of eggs per unit volume was increased. This same dependence on egg concentration was found for the effects of various nitro and halophenols on *Arbacia* and for the effect of dinitrocresol on *Chaetopterus.* These effects are just the reverse of those observed when anesthetics are employed to block division. The action of high egg concentrations with nitro and halophenols was proved to be due to the accumulation of respiratory carbon dioxide.

Experiments with added CO_2 showed: (1) A CO_2 tension, which alone had a relatively slight adverse effect on division, intensified the dinitrocresol division block and the magnitude of this effect was more than the sum of the separate effects for the individual reagents. (2) Concentrations of CO_2 from .5 per cent to 2 per cent intensified the respiratory stimulating effects of sub-optimum concentrations of dinitrocresol. (3) In a similar way, carbon dioxide intensified the division block and accentuated the respiratory stimulation obtainable with sub-optimum concentrations of paranitrophenol, 2, 4 dinitrophenol and 2, 4 dichlorophenol.

Further studies on the effect of numbers present on the rate of cleavage in Arbacia. W. C. Allee and Gertrude Evans.

Paradoxical osmotic volume changes in erythrocytes. A. K. Parpart and M. H. Jacobs.

Earlier work by the authors (Biological Bulletin, **65**: 513, 1933) indicated that erythrocytes exposed to solutions of non-penetrating non-electrolytes undergo a reversible decrease in their internal osmotic pressure through an exchange of anions from the cell for OH′ ions from the solution, with a consequent increase in the proportion of the total base bound by hemoglobin. Direct evidence, however, was lacking that under these conditions reversible volume changes of the cells occur. This evidence has now been supplied, partly by hematokrit measurements and more clearly by the photoelectric method described elsewhere by one of the authors (Parpart). Appropriate experiments show that in solutions of sucrose which from their freezing points would be expected to be isotonic or even slightly hypotonic to the cells there is a rapid decrease in the volume of beef and rat erythrocytes. The addition of small quantities of salts to suspensions of erythrocytes in such sucrose solutions at first causes a further shrinkage of the cells due to the increased osmotic pressure of the solution, but unless the electrolyte concentration be too great, this shrinkage within a few seconds is followed by a considerable swelling. The optimum concentration of NaCl for producing swelling is about 0.005 M, which is approximately the same as that already found in unpublished experiments to cause the greatest increase in osmotic hemolysis in hypotonic sucrose solutions.

Further studies on specific physiological properties of erythrocytes. M. H. Jacobs, H. N. Glassman and A. K. Parpart.

It was previously observed by one of the authors (Parpart) that the erythrocytes of the ground-hog are remarkably permeable to certain non-electrolytes of relatively high molecular volume such as erythritol, mannitol, xylose, glucose, thiourea, etc. These observations have been confirmed by a further study of the blood of 3 more animals of the same species, and additional determinations have also been made of the permeability of the erythrocytes of the ground-hog and of 6 other species of mammals to a series of 15 ammonium salts. In general, the erythrocytes of the ground-hog have been found to be no more permeable to the ammonium salts studied than are those of the other species, except in the case of ammonium borate to which they seem to show a unique permeability. Experiments designed to give evidence by osmotic means of leakage of salts from the erythrocytes also failed to show a more rapid escape from those of the ground-hog than from those of several other species. Evidently, therefore, a very high permeability to non-electrolytes is not necessarily associated with a high permeability to electrolytes. In the case of the blood of the rat, two apparently specific characters, not found with the other species studied, have been investigated. They are (a) the complete failure of ammonium benzoate to retard hemolysis by ammonium chloride and (b) a very high resistance to hemolysis by distilled water after a previous exposure to a hypertonic salt solution. The blood of the skunk (3 individuals) has also been investigated. In addition to certain other less striking specific properties, it apparently differs from that of the other species studied in undergoing rapid hemolysis in a 4 : 1 mixture of M/6 NaCl and M/6 $NaHCO_3$.

August 28

Experiments on the contractile substance of muscle fibers. C. C. Speidel.

Retraction caps of injury may be induced readily in the striated muscle fibers of living frog tadpoles, if these are orientated properly and subjected to electrical stimulation of suitable strength. A primary retraction cap appears first at the muscle-tendon junction. It is a coagulation product, or muscle clot, which involves a few or many sarcomeres of the contractile substance. It quickly stabilizes the injured end of the fiber, prevents loss of tension, and thus makes for the preservation of the remainder of the fiber.

Further electrical treatment usually causes the contractile substance to separate from the retraction cap. As it draws back a second cap forms. If the fiber is long enough, third and fourth caps may arise in similar fashion until ultimately the entire fiber has undergone complete retraction. In the case of long fibers, such as those teased from the gastrocnemius muscle of the adult frog, multiple retraction caps (a dozen or more) may be induced successively. If, however, muscle fibers in the tadpole's tail are orientated with their long axes at right angles to the direction of the current, retraction caps do not form.

Retraction caps may be induced on young regenerating fibers any time after the differentiation of cross striæ, but not before. Retraction caps do not form in either smooth muscle or heart muscle.

A wide variety of methods (and reagents) has been employed in investigating the response of the contractile material, which supplements the data from electrical stimulation. Many of the phenomena have been recorded by means of ciné-photomicrography.

The injection of aqueous solutions, including acetylcholine, into the isolated muscle fiber. Elsa M. Keil and F. J. M. Sichel.

Very small amounts (5,000 μ^3) of distilled water, Ringer's solution, isotonic sucrose or sodium chloride solutions, when injected into, or blown upon the surface of the isolated single muscle fiber (adductor muscles of frog's leg), produce no visible effects apart from a purely mechanical passive disturbance. This has been found to be true only if a clean, previously unused, or freshly washed micropipette is employed. If a larger quantity is injected or if the injection is done too rapidly, or if a previously used pipette is employed, a localized contraction of the interior of the fiber occurs, which is immediately reversible on stopping the injection. Coincident with the contraction the fibrillar structure becomes sharply visible at the tip of the micropipette. The contraction is accompanied by the diminishing of the inter-striæ distances.

The application or injection of $CaCl_2$, as pointed out by Chambers and Hale, causes a violent shortening of the fibrillar material. This shortening is reversible and occurs even when minimal amounts of M/200 solution are injected.

The application or injection of acetylcholine in concentrations ranging from 1 in 10^3 to 1 in 10^{11} has no effect on the isolated fiber, even when eserinized, beyond the effects described above attributable to distilled water, Ringer's solution, etc.

The fiber preparation is capable of giving a twitch response to electrical stimulation although it is incapable of giving a conducted response, and is apparently denervated. Effects of acetylcholine in concentrations within the range here used have been reported in perfused striated muscles. We conclude that such action is not upon the contractile mechanism directly.

The double refraction of smooth muscle. E. Bozler.

The double refraction of smooth muscle and its changes during contraction are studied by a new method. By means of a mica wedge in front of the analyser, fringes are produced, the position of which indicates the double refraction of any part of the muscle. In some preliminary experiments it was found that, on stretching, the double refraction of the muscle (retractor of the snail *Helix pomatia*) increases linearly with the square root of the length. This change cannot be regarded as a photo-elastic effect because no tension is present within the range of lengths used. The effect produced by increasing the length is probably due to the orientation of molecules previously unoriented. It was furthermore found that during an isometric contraction there is no change of double refraction within the limits of the accuracy of the method (1–2 per cent). The contrary result obtained for smooth muscle may be explained by the fact that the contractile segments of the myofibrils are able to shorten even in an isometric contraction. There is no evidence that production of tension in muscle is associated with a change of double refraction.

Some physical and chemical properties of the axis cylinder of the giant axons of the squid, Loligo pealii. F. O. Schmitt, R. S. Bear and J. Z. Young.

Because of their large size the giant axons of the squid are well suited for the investigation of the physical and chemical properties of typical axis cylinder. The birefringence is, as usual, positive with respect to the length of the fiber, in this case being 5×10^{-5}. In alcohol and certain other reagents, the axis cylinder shrinks from the surrounding sheaths to less than 50 per cent of its original diameter and may be withdrawn as a single strand retaining considerable birefringence. By using the immersion technique this double refraction was shown to be largely of form character, though a considerable amount of intrinsic micellar contributions is present.

The axis cylinder proteins exhibit striking shrinkage and swelling changes of an osmotic type. These properties may be observed even after fixation for hours in formalin and must be considered in connection with histological methods.

By extruding the axis cylinder material into small volumes of water or salt solution it is possible to investigate the protein components of pure axoplasm. It was found that the principal protein of this material corresponds to that extracted by neutral solutions from lobster claw nerves by Schmitt and Bear. The extrudate dissolves almost completely in distilled water, but the solubility is markedly increased by the presence of salts. Though in this respect the protein resembles the globulins, the fact that addition of acetic acid produces a flocculent precipitate, which is again soluble in dilute alkali, suggests instead that it is a nucleoprotein. Certain other chemical evidence obtained in connection with attempts to prepare nucleotides from this protein offer confirmation, but complete proof awaits experiments to be carried out with the more abundant lobster nerve material.

The structure of the eye of Pecten. G. Schoepfle and J. Z. Young.

To assist in Dr. Hartline's investigation of the action potentials in the optic nerve of *Pecten irradians* we have re-examined the structure of the eye, using various general histological and special neurological techniques. As has been shown by Dakin, Küpfer and other workers the retina contains two distinct layers of sensory cells, each giving rise to a nerve. The two nerves run separately for some distance before uniting behind the eye.

In Golgi preparations it is easy to see that the processes of the elongated cells of the proximal layer pass into the nerve without the intervention of any synapse. In the case of the more rounded cells in the distal layer the connections are less easy to make out, but in favorable cases it can be seen that axons arising from the cells pass directly into the nerve which arises from this part of the retina. While it is possible that there are other types of cell present which have not been stained in our Golgi preparations, yet since we have never seen any indication of them it seems probable that both the proximal and the distal layers consist only of primary sense cells and interstitial cells, and that there are no other nerve cells, and no synapses in the eye.

A further point of interest is that the fibers of the common optic nerve, formed by fusion of the branches arising from the two layers, run across the ring nerve which runs around the edge of the mantle, and pass directly into the radial pallial nerves. The optic nerve bundle sometimes runs with the ring nerve for a short distance but certainly the great majority, and probably all of its fibers pass on into one of the pallial nerves without passing around the mantle for any great distance.

The discharge of impulses in the optic nerve fibers of the eye of Pecten irradians. H. K. Hartline.

The discharge of impulses in the optic nerve fibres of the eye of the scallop, *Pecten irradians*, has been studied by recording their amplified action potentials. In the whole optic nerve the response to illuminating the eye is strongest at the beginning of illumination, diminishes distinctly after several seconds, but nevertheless continues as long as the light shines. When the light is turned off there is another strong outburst of nerve impulses, lasting several seconds.

It has been possible to study separately the responses of the two different layers of sensory cells in the *Pecten* eye, since the fibres from each layer form distinct branches of the optic nerve.

Impulses are discharged in the fibres from the proximal sensory cells only when the eye is illuminated. They cease when the light is turned off. Records from single fibres show a regular series of impulses beginning at a high frequency and adapting fairly rapidly to a level which is maintained as long as the light shines. The level of frequency is higher the greater the intensity of illumination.

Impulses are discharged in the fibres from the distal sensory cells in response to cessation of illumination. This discharge may last several seconds, or even minutes; it is abruptly stopped if the eye be re-illuminated. Records from single fibres show that the frequency of the discharge is initially high, but diminishes rapidly. The level of frequency and the duration of the discharge are greater the more intense and more prolonged the preceding illumination. In some cases, following strong illumination, it has been observed that the discharge in these fibres may break up into rhythmic bursts of impulses, the bursts occurring synchronously in all the fibres of this branch of the optic nerve.

The effect of light on the CO-poisoned embryonic Fundulus heart. K. C. Fisher and J. A. Cameron.

When embryos of trout, salmon, or *Fundulus* are subjected to mixtures of CO and O_2 dissolved in water the frequency of the heart falls below the normal value by an amount depending on the ratio of the concentrations of O_2 and CO, and the inhibition is relieved by visible light to an extent depending on the intensity of the illumination. The behavior found suggests that in the presence of CO the frequency of the heart depends on the ratio of the CO-free and CO-combined fractions of Warburg's "atmungsferment." Light causes the dissociation of the CO-ferment complex, more O_2 is then able to pass through the system and the beat frequency recovers. If this interpretation is correct, it should be possible to replace "O_2 consumption" in Warburg's theoretical development by "heart beat frequency." Using the formula $\frac{\text{uninhibited frequency}}{\text{inhibited frequency}} = K \frac{[O^2]}{[CO]} I$ we have partially tested this possibility using *Fundulus* embryos, and, as required by the equation, find that at a fixed $\frac{[O_2]}{[CO]}$ the $\frac{\text{uninhibited}}{\text{inhibited}}$ is a function of I, the light intensity. The slopes of the graphs of "$\frac{\text{uninhibited}}{\text{inhibited}}$ against I lines" vary in the expected direction with changes in $\frac{[O_2]}{[CO]}$, though the data are not yet complete enough to be quantitative in this respect. The time course of the frequency change upon illumination of a CO-poisoned preparation is that of a first order process, which process is probably not the decomposition of the CO-ferment compound.

We conclude that there is a reactant interposed between the "atmungsferment" and the rhythmic production of stimuli to the heart muscle. Change in the light intensity changes the concentration of this reactant as indicated by the frequency of the heart and it is this reactant whose change in concentration is an apparent unimolecular chemical reaction.

Prevention of edema in frog perfusions in the absence of serum proteins. G. Saslow.

Drinker has observed that leakage through frog capillaries can be prevented for over 3 hours by the addition of a minimum of 15% of horse serum to a perfusion liquid such as 3% acacia in 0.65% NaCl. The acacia alone is ineffective, even if enough be used to make the oncotic pressure three times that of frog blood. Drinker concludes that serum possesses a peculiar power of restraining capillary leakage.

In preliminary experiments on this subject, it was observed that the web of the foot of perfused frogs developed no edema in 5 hours (when the experiments were terminated) when either of the following solutions was used:

1. 5% gum acacia
   0.014% KCl
   0.75% NaCl
   P (NaH_2PO_4, Na_2HPO_4), 10 mg./100 cc.
   23% beef red cells (washed twice)
   pH 7.2
2. The same as 1, except that the acacia concentration was 3%.

During perfusion the solutions were continuously stirred with oxygen from a tank.

It is thus clear that if oxygenation be adequate, the serum constituents can be replaced completely by a colloidal polysaccharide in such concentration as to give approximately the same viscosity and oncotic pressure to the fluid. That serum possesses any special power of restraining capillary leakage, under normal circulatory conditions, appears quite improbable. These conclusions are in complete accordance with those already published by Amberson, Stanbury and co-workers for mammals.

Behavior of frog tadpole epidermal cells during seven successive regeneration periods. J. A. Cameron and K. O. Mills.

Bullfrog tadpoles about 5 cm. long were kept at high temperature and exposed to alternate twelve-hour periods of darkness and intense visible light. Every twenty-four hours a 5 mm. piece was cut from the tail of each experimental animal. The plane of the cuts was at right angles to the tail-axis. The seventh cut was usually fatal, but a few lived until the ninth day. Histological study showed that after each injury the epidermis covered the exposed wound surface by migration from the region anterior to the cut. Since on each successive day a new and more extensive injury than that of the day preceding stimulated new migration, it might have been expected that mitosis would increase with the severity and duration of the demand for new cells. In no case did the rate of mitosis in the skin of the regenerating animal equal that of the controls or of the first specimen from the experimental animal concerned. The counts included all the skin of the parts cut off and almost all the mitoses found were in lateral, non-migrating cells. The sample for each day showed a definite decline in the mitotic rate as compared with the rate for the preceding day.

Two explanations are suggested: one, that each injury induced or contributed to the production of an inhibition of mitosis in the skin which extended at least as far as 5 mm. anterior to the cut and lasted for at least twenty-four hours; the other, that cells lost through migration are not in all cases replaced by mitosis of epidermal cells but by addition and differentiation of traveling cells from the connective tissue of the dermis.

Observations on conditions affecting growth of cells and tissues, from microscopic studies on the living animal. E. R. Clark and Eleanor L. Clark.

A careful microscopic study of the growth and differentiation of new tissue in many double-walled transparent chambers installed in rabbits' ears, in which the tissue has been subjected to a variety of experimental conditions, has revealed the following.

The inflammatory exudate which fills the space within the first 24 hours after installation, consisting chiefly of blood plasma, erythrocytes singly or in clumps, fibrin and leucocytes, all in varying proportions, promotes rapid growth of cells from surrounding tissues. If the chamber is subjected to continued insults, so that there are repeated renewals of the inflammatory exudate, new growth continues until the space is completely occupied by new tissue. If, however, the chamber is protected from insults so that no appreciable renewal of the inflammatory exudate occurs but only the normal interchange between blood and outside intervascular substance, the rate of growth gradually diminishes. Thus in a chamber demonstrated at this session, in which the maximum success has been achieved in eliminating insults, such a slowing-down of growth occurred that, in the central part of the chamber, a lake of semi-fluid material filling more than half the thickness of the space has remained for a month uninvaded by fibroblasts or blood-capillaries.

It seems obvious that, to induce growth of cells there must be present in their immediate environment growth-promoting conditions which involve both physical and chemical properties. The mere presence of a space filled with fluid or semi-fluid material and undoubtedly containing proteins, is not sufficient to induce growth of fibrous or vascular tissue.

Incidentally, the lymphatics which vary in different specimens have been carefully studied in many chambers, and it has been found that the rate of growth of fibroblasts, blood capillaries, etc., shows not the slightest relation to the presence or absence of functional lymphatics.

Some nuclear phenomena in the Trichodina (Protozoa, Ciliata, Peritrichida) from Thyone briareus (Holothuroidea). Laura N. Hunter.

The Trichodina occurring in great numbers in the gut of *Thyone* exists in two forms. Type I inhabits the anterior secretory portion of the gut, type II the posterior absorptive region. Type II individuals are elongated, flattened, possess few pellicular wrinklings and contract seldom. Type I individuals are bag-shaped, possess pellicular annulations as do certain vorticellids and contract frequently. One hundred type I individuals averaged 112 microns long by 75 microns wide as contrasted to 78 by 50 microns in 100 type II individuals. The square root of the average length times width of the corona, a skeletal ring in the adhesive disc, was 51 for 100 type I and 37 for 100 type II individuals. The vegetative macronucleus is elongated and somewhat E-shaped in type I and bean-shaped and compact in type II. In both types it appears vacuolated and heterogeneous,—becoming homogeneous as fission approaches. The resting micronuclei of both forms show striations suggestive of chromosomes. Binary fission occurs similarly in both forms but the early post-fission macronucleus in type I is elongated and bent in contrast to the compact bean-shaped post-fission macronucleus in type II. The micronuclei of both forms divide mitotically, with chromosomes and spindle fibers clearly visible. Association between a large and a small individual also occurs in both types. It is accompanied by macronuclear fragmentation and disintegration and several mitotic divisions of the micronucleus. The exact process is not yet worked out but during the ensuing reorganization the larger individual—in type II at least—contains successively 4 micronuclei and 4 partly granular macronuclear anlagen, then 1 micronucleus and 4 macronuclear anlagen. Cells containing 2 macronuclear anlagen and 1 micronucleus presumably result from a cell division which separates the macronuclei while the micronuclei divide mitotically. The 2 macronucleate stage shows macronuclei free from chromatic granules.

Investigations on determination in the early development of Cerebratulus. S. Hörstadius.

In the 16-cell stage the egg of the nemertine *Cerebratulus lacteus*, which undergoes a spiral cleavage, consists of four layers of each four cells, which layers we may designate as an_1, an_2, veg_1, and veg_2. Vital staining of the animal half shows that this material gives rise to the ectoderm of the Pilidium-larva, inclusive of a great part of the ciliated band. The vegetative half forms the stomach, oesophagus, the inside of the two lappets, and a part of the ciliated band. veg_2 constitutes the material for the stomach, an_1 corresponds to the greater, most animal part of the ectoderm probably including a small piece of the ciliated band.

Several authors, E. B. Wilson, Zeleny, and Yatsu have found that fragments cleave as fragments, and also, to some extent, shown that they differentiate as fragments. This was confirmed in greater detail. Animal halves have apical organ and ciliated band but no archenteron; vegetative halves have no apical organ, but a large archenteron and a small ectoderm with a ciliated field. $an_2 + veg_1 + veg_2$ has no apical organ and the ectoderm is too small. In $an_1 + an_2 + veg_1$ the stomach is missing, but we find under the ciliated band a wall, corresponding to the oesophagus and the inside of the lappets. an_1 isolated develops into a blastula with an apical organ and a minute piece of ciliated tissue (compare above!), whereas veg_2 isolated dies at a late cleavage stage. $an_2 + veg_1$ differentiates as the middle part of the larva, with a broad ciliated band.

Also when fusing an_1 with veg_2 the fragments develop as they would have done normally: the larva lacks a large ciliated band and oesophagus. The same holds for an animal half transplanted to a meridional half: each component differentiates in accordance with its prospective significance; they seem not to exert any influence upon each other, as is so markedly the case in the sea-urchin egg. The early cleavage stage of *Cerebratulus* thus belongs to the mosaic type of development. But in the uncleaved egg both the animal and the vegetative halves can form complete larvae.

Preliminary evidence as to a source of the growth and the sex-stimulating hormones in the bullfrog. R. Rugh.

Mechanism of hatching in Fundulus heteroclitus. P. B. Armstrong.

Two factors operate to induce hatching in *Fundulus* eggs: (1) the lashing movements of the tail of the embryo, and (2) an enzyme liberated by the developing embryos. The operation of the first of these can be shown by abolishing body movements with chlorotine or KCl at the time when hatching normally should occur. In the absence of the body movements there is no hatching. The operation of the second factor can be shown by treating the eggs immediately after fertilization with 4 per cent to 5 per cent alcohol for the first two days of development. In eggs so treated, some embryos are found with a symmetric menophthalmia and a long, snout-like and sometimes non-patent mouth. In such embryos the hatching enzyme which is produced within the mouth and pharyngeal cavities does not get out into the perivitelline space where it can act on the membrane, and the body movements alone, which appear unimpaired, cannot rupture the chorionic membrane.

The enzyme-bearing cells are seen within both the mouth and pharyngeal cavities. They are unicellular and are full of a granular eosinophilic substance which crowds the nucleus to the periphery of the cell. After hatching, these cells disappear completely.

Hermaphroditism in Mollusca. B. H. Grave and J. Smith.

A study of fifteen species of mollusks has been made during the past summer by the method of paraffin sections, with the object of ascertaining whether hermaphroditism is the rule or the exception among the commonest local species. The selection was made to represent as many families as possible, but those chosen were chiefly lamellibranchs. The Amphineura are represented by *Chaetopleura apiculata*, and the Gastropoda by two marine and two fresh-water species, *Urosalpinx cinereus*, *Nassa obsoleta*, *Planorbis trivolvis*, and *Succinea ovalis*, respectively. Our findings in the main confirm the generalization by Pelsener (1910) that hermaphroditism reaches its highest expression among the most highly specialized forms, such as the oyster, the scallop, and the shipworm. However, some degree of bisexuality is common, varying from that scarcely detectable, to pronounced hermaphroditism. Some lamellibranchs in which the sexes are quite separate in the adult, have hermaphroditic gonads during the early stages of sex differentiation. Some of the latter are protandric, and some develop from the beginning into males and females, the gonads showing some degree of hermaphroditism. This is especially true of the males. In fact, the testes of young individuals of many species of lamellibranchs contain small and sometimes well developed oögonia as well as mature spermatozoa. The gonads of *Venus mercenaria*, for instance, contain well developed oögonia and spermatozoa, at least during the first year's growth. Loosanoff (1936) describes it as protandric, but the sexes are separate in the adult. Young males of *Chaetopleura apiculata* contain small oögonia which later disappear. Other individuals of this species are females from the beginning of sex differentiation. The sexes appear to be entirely separate in the adult and practically so in the young.

During the breeding season the sexes appear to be separate in the following species, and there is no sign of sex inversion: *Yoldia limatula*, *Anomia simplex*, *Ensis directus*, *Mytilus edulis*, *Modiolus demissus*, *Mya arenaria*, *Cumingia tellinoides*, *Venus mercenaria*, and *Chaetopleura apiculata*. The young stages of these species have not been studied, with the exception of *Venus mercenaria* and *Chætopleura apiculata*, as described above. In all these forms the sexes are separate in the adults if not throughout life.

Pecten irradians and *Lævicardium mortoni* are truly hermaphroditic, having a functional testis and a functional ovary at the same time. Self-fertilization takes place in this species of *Pecten* as was shown by a series of experiments which resulted in normal development when cross-fertilization was prevented by isolation. Self-fertilization may take place also in *Lævicardium*, although experiments with this animal were not attempted.

There is a change in sex in *Teredo navalis* from male to female, and vice versa. This species is not truly hermaphroditic in that the gonad does not function as an ovary and a testis at the same time but alternately.

In *Planorbis trivolvis* and *Succinea ovalis* an ovatestis is present. The alveoli give rise to both eggs and sperm at the same time. These snails appear to be permanently hermaphroditic. Hickman (1931) gives the breeding season of *Succinea ovalis* as April to September and states that after the hibernating period spermatozoa and eggs develop at the same time, an example of "simultaneous hermaphroditism."

In conclusion it may be said that many species of mollusks appear to be dioecious, but when sexual cycles have been studied adequately, especially during early sex differentiation, some classed as dioecious may be shown to be hermaphroditic or protandric. Sex in many species is not fixed but may be altered both by external and internal changes. The indications are that it may be possible to induce changes in sex in mollusks experimentally.

Papers Read by Title

Separation of the conducting and contractile elements in the retractor muscle of Thyone briareus. H. G. duBuy.

The retractors which are attached to the calcareous ring of the lantern of *Thyone briareus* present a type of muscle which is intermediate between vertebrate striated and vertebrate smooth muscle. The histological characteristics of these retractor muscles and their sensitivity to choline-esters and adrenalin can be compared with properties of vertebrate smooth muscle; their sensitivity to curare and the effect of this drug on the contractile elements can be compared with properties of vertebrate striated muscle.

It can be shown that the action potentials obtained from the nerve-muscle preparation of *Thyone* occur only when the conducting elements are not blocked (for example by curare). After curarisation no action potentials occur while local contractions are still obtainable. These phenomena allow a further differentiation between the properties of the conducting and the contractile elements.

After curarisation of a part of the muscle, the interesting phenomenon of a distal contraction of the non-curarised part can be observed. This is similar to the phenomenon which was first described by Steiman for the single muscle fibers of the frog's tongue. When an electric stimulus is applied to the curarised part of the muscle of *Thyone*, only the non-curarised distal part of the muscle shows a contraction which can be more or less local. After an increase of the intensity of the electric stimulus both the local contraction under the stimulating electrodes in the curarised part, and the distal contraction of the non-curarised part can be obtained. Therefore the threshold for electric stimulation of the conducting elements is markedly lower than the threshold of the contractile elements.

The application of curare prevents the occurrence of spontaneous contractures, etc., which often have hampered investigation of the plastic properties of smooth muscles. Curare does not seem to change these properties. Some differences, however, occur in the extension-curve obtained by means of weights. These differences can be explained by the absence of the activity of the conducting elements.

Hyperparasitism: A species of Hexamita (Protozoa, Mastigophora) found in the reproductive systems of Deropristis inflata (Trematoda) of marine eels. A. V. Hunninen and R. Wichterman.

A species of *Hexamita* was discovered parasitic in the reproductive organs of the trematode, *Deropristis inflata*, found in the marine eel, *Anguilla chryspa*. Of 154 eels examined, 41 were infected with the trematode; 15 of these infected eels harbored 80 trematodes which contained the protozoan parasites.

Measurements showed the length of the protozoön to be 10.2 μ and the width 5.2 μ; the animal being rounded anteriorly and pointed posteriorly. Six flagella originate from the anterior end, being approximately of the same length as the body and directed posteriorly. The two posterior flagella which are approximately twice the length of the body, continue from the axostyles. Two compact club-shaped nuclei are located in the anterior end of the flagellate and extend slightly more than one-third the body length.

The flagellates were limited exclusively to the reproductive organs of the trematode, especially to the female system. They were found in large numbers in the eggs, uterus, oviduct and seminal receptacle. In a few cases they were observed in the vitelline glands and testes. What seemed to be noteworthy was the continued occurrence of the parasites in varying numbers in the eggs of the trematode. In several instances as many as 20 were found in one egg. The protozoa live at the expense of the protoplasmic material of the egg which would normally give rise to a miracidium. In some cases all the eggs in the uterus were parasitized by *Hexamita*, completely preventing miracidial development. The conspicuous absence of *Hexamita* from the intestine of the eels strongly points to a rigid host-parasite specificity between the protozoön and the trematode since flagellates were found in only 2 of the 141 eels examined.

Members of the genus *Hexamita* are of interest because of their widely diversified associations in nature. Some species of the genus are free-living and others associated with vertebrate and invertebrate hosts. This appears to be the first observation of trematodes being parasitized by these flagellates. Further details with figures will be presented in the complete paper.

Cytological studies of the genus Chilodonella. III. The conjugation of Chilodonella labiata, variation? Mary S. MacDougall.

Results of studies of the effects of ultraviolet variation on *Chilodonella* made an investigation of the cytology of this genus desirable. Previous work has shown that the six species whose conjugation and division phenomena have been studied fall naturally into three groups:

1. The Cucullulus group.
2. The Labiata group.
3. The Uncinata group.

Each of these groups has certain definite characteristics peculiar to it, especially as to behavior in conjugation and division.

In Stuart's Pond, a brackish body of water in Woods Hole, Mass., a *Chilodonella* was recently found which looked more like Stokes' figures of *C. caudata* than the fresh-water form of this species. The so-called tail, however, was not constant, and the living material was often confused with *C. uncinata.* The macronucleus is centrally located, however, as it is in *labiata,* and the details of conjugation are identical with that form, i.e. the macronucleus as well as the micronucleus is exchanged during conjugation. There are several points of difference between the new form and *labiata* which seem to be constant. While it is definitely not the typical *labiata* recently described by the author, it is evidently a variation of this type.

It has been found that in nature there is a series of closely intergrading forms in this genus, and further genetic work must wait on more complete knowledge of the cytology of this group.

Comparative hypotonic cytolysis of several types of invertebrate egg cells and the influence of age. Victor Schechter.

The resistance to cytolysis in distilled water was studied for several species of unfertilized invertebrate egg cells. The figures below give the time required for fifty per cent of the cells to cytolyze.

The egg cells of *Mactra solidissima* showed a drop from approximately 8½ minutes when fresh to 3 minutes when 20 hours old. For *Cumingia tellinoides* the corresponding figures were 8 and 3½ minutes. The egg cells of *Heteronereis limbata* showed a resistance of 6½ minutes when fresh and a drop to 4¾ minutes when 28 hours old. For *Echinarachnius* and *Asterias* sufficient quantitative data are not yet available but a similar trend is indicated. Freshly shed *Arbacia* egg cells cytolyze in 3½ minutes.

What significance, if any, lies in the initial difference in resistance of the fresh eggs of these various forms is not yet apparent. In 1932 (Goldforb and Schechter, *Proc. Soc. Exp. Biol. and Med.*) a progressive decrease in resistance to hypotonicity by the ageing egg cells of *Arbacia* was demonstrated. The extension of this finding to other species weights rate of cytolysis as a factor of some importance in the process of ageing. In view of the fact that hypotonic cytolysis is a complicated phenomenon involving the interior of the egg as an osmotic system and also the physical and chemical properties of the cell surface, it is of special interest that all eggs so far studied show a similar trend with age. Preliminary experimental analysis has been made and will be presented in an early paper. It may be of interest to report at this time that, for *Arbacia* egg cells, pretreatment with excess calcium increases resistance to hypotonic cytolysis, decreased calcium lowers resistance and similar solutions also affect the duration of life.

Notes on life cycles of digenetic trematodes. H. W. Stunkard.

Experiments, conducted under carefully controlled conditions, have given the sexually mature stages of two species of larval trematodes from *Nassa obsoleta.* Both species were studied and named by Miller and Northup (1926).

One species, *Cercaria quissetensis*, occurs in about one per cent of the snails examined. The cercariae swim about near the bottom and encyst in various species of mollusks. Experimental infection of *Mya arenaria*, *Mytilus edulis*, *Modiola modiolus*, *Cumingia tellinoides*, *Pecten irradians*, *Ensis americana* and *Crepidula fornicata* was obtained. Metacercariæ recovered three days after encystment were infective for gulls and developed into adults of the species identified by Linton (1928) as *Himasthla elongata* (Mehlis). Constant differences between these specimens and the description of *H. elongata* were observed by Linton and it appears that the worms belong to a distinct species, *Himasthla quissetensis*, first named in the larval stage by Miller and Northup. The specimens found in natural infections are identical with those obtained in experimental ones.

Cercariæum lintoni of Miller and Northup is identical with *Distomum lasium* Leidy, 1891. These larvae encyst in the parapodia of *Nereis virens* and other annelids. After three weeks in the second intermediate host, the metacercariæ were fed to various fishes and developed to sexual maturity in the intestine of the eel, *Anguilla chrysypa*, and the toadfish, *Opsanus tau.* The worms persisted for two to three weeks in the intestine of *Paralichthys dentatus* and *Tautoga onitis*, and it is probable that they reach maturity in these species also. Natural infections were found in the eel. The adult worms are specifically identical with *Zoögonus rubellus* (Olsson, 1868), (synonym, *Zoogonus mirus* Looss, 1901).

Inhibition of gastrulation in Arbacia with Nickel Chloride. A. J. Waterman.

For several summers a study has been made of the effect of various physical and chemical agents upon gastrulation in the sea-urchin, *Arbacia punctulata.* Gastrulation in this form is much more resistant to experimental manipulation than in *Paracentrotus*, while the embryos of the sand-dollar and starfish are particularly sensitive. The chlorides of Na, K, Mg, and Ca alone or in combination, hypotonic and hypertonic sea water, variation in temperature, X-ray and alcohols have been tried.

Recently the blastula and young gastrula stages have been exposed for many hours to various concentrations of some of the metallic chlorides. Of these, certain concentrations of a 1 per cent solution of $NiCl_2$ in sea water have given numerous, excellent exogastrulæ which grew and differentiated a fairly typical body form, skeleton, external gut and stomodæum. The types are not quite as good as those obtained previously with *Paracentrotus* but are the best that have thus far been secured with *Arbacia.* Nickel chloride seems to have a differential effect upon gastrulation in some individuals but the embryos of a culture vary in their susceptibility. Some show total inhibition of any endoderm formation and grow as large, oval vesicles with differentiated ectoderm, apical plate, stomodæum and irregular apron-like growths around the edge of the former vegetal pole region. Nickel chloride also produces skeletal modifications, as well as inhibition and retardation of development depending upon the concentration. A comparison of the effects upon the blastula and young gastrula stages seems to show little or no differential susceptibility previous to or during gastrulation. Gastrulation may be temporarily inhibited for many hours. The general effects upon development, except for exogastrulation, are very similar to those observed with the alcohols and other agents listed above.

Division and conjugation in Nyctotherus cordiformis (Ehr.) Stein (Protozoa, Ciliata) with special reference to the nuclear phenomena. R. Wichterman.

A cytological study of the complete life cycle of *Nyctotherus cordiformis* (Ehr.) Stein, a heterotrichous ciliate from the large intestine of tadpole and adult tree toads, *Hyla versicolor*, was studied. In division, a partial dedifferentiation of parental ingestatory structures occurs, then redifferentiation to be retained by the anterior daughter individual. For the posterior daughter, the ingestatory apparatus arises *de novo*. The macronucleus divides amitotically while the micronucleus divides mitotically, demonstrating small bead-like chromosomes. Dividing animals may be found in tadpole and adult hosts. However, during metamorphosis of the tadpole into adulthood, divisions of the ciliates result in small forms, the pre-conjugants, which are the only ones capable of conjugation. Therefore conjugation takes place only during the metamorphosis of the host. In conjugation, two ciliates fuse along their peristomes. The macronucleus undergoes complete fragmentation while the micronucleus of each conjugant divides three times. The first pregamic division results in two micronuclear products; the second division, in four micronuclear products, three of which degenerate. The remaining product enters into the third pregamic division to produce the two functional migratory and stationary pronuclei. Interchange of migratory pronuclei follows at the fused anterior ends of the conjugants to form the amphinucleus which divides once to produce the micronucleus and the macronuclear anlage. Development and behavior of the unusual macronuclear anlage ("spireme ball" of Stein and Schneider) was observed for the first time.

Ex-conjugants were found nearly exclusively in recently transformed hosts. While the animals remain fused in conjugation for less than a day, the reorganization process of the ex-conjugants takes several weeks. Toward the end of the conjugation process ingestatory structures of each conjugant completely dedifferentiate while a new set arises *de novo* posterior to the old ones.

Conjugation here is interpreted as an effort made by the ciliates to overcome a physiological crisis during the drastic host transformation changes.

Note: The complete paper will appear in the *Journal of Morphology.*

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CONTENTS

Page

PARKER, G. H.
The Reactivation by Cutting of Severed Melanophore Nerves in the Dogfish Mustelus 255

ABRAMOWITZ, A. A.
Physiology of the Melanophore System in the Catfish, Ameiurus 259

SMITH, G. M., AND C. W. COATES
Cutaneous Melanosis in Lungfishes (Lepidosirenidae) 282

COWLES, R. P., AND C. E. BRAMBEL
A Study of the Environmental Conditions in a Bog Pond with Special Reference to the Diurnal Vertical Distribution of Gonyostomum semen 286

HEILBRUNN, L. V.
Protein Lipid Binding in Protoplasm 299

MAZIA, DANIEL, AND JEAN M. CLARK
Free Calcium in the Action of Stimulating Agents on Elodea Cells 306

ZOBELL, CLAUDE E., AND D. QUENTIN ANDERSON
Observations on the Multiplication of Bacteria in Different Volumes of Stored Sea Water and the Influence of Oxygen Tension and Solid Surfaces 324

TRAGER, WILLIAM
The Utilization of Solutes by Mosquito Larvae 343

COE, W. R.
Environment and Sex in the Oviparous Oyster Ostrea virginica 353

GRAFFLIN, ALLAN L.
Renal Function in Marine Teleosts. IV. The excretion of inorganic phosphate in the sculpin 360

KOONZ, CARL H.
Some Unusual Cytological Phenomena in the Spermatogenesis of a Haploid Parthenogenetic Hymenopteran, Aenoplex smithii (Packard) 375

PROGRAM AND ABSTRACTS OF SCIENTIFIC MEETINGS, MARINE BIOLOGICAL LABORATORY, 1936 386

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THE

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THE EFFECT OF ALCOHOL ON GASTRULATION IN ARBACIA

A. J. WATERMAN

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I

Following certain changes in the environment, the endoderm of echinoderm embryos (*Arbacia*, *Paracentrotus*, *Asterias*, *Echinarachnius*) can be made to develop externally as an exogastrula. Other effects include the development of the endoderm at the expense of the ectoderm and the irregular formation of skeleton and body form. First discovered by Herbst as a lithium effect, exogastrulation has since been shown by several investigators to be caused by a large variety of agents, both chemical and physical (cf. Waterman, 1934). The result is given by fertilized eggs, cleavage stages or blastula stages. Recently exogastrulation has been provoked in another group of animals, the Amphibia. Holtfreter (1933) has found that exposure of axolotl eggs to NaCl solutions caused exogastrulation in some of the embryos, while Curtis et al (1936) have provoked exogastrulation by exposing blastulae of *Amblystoma*, *Rana* and *Bufo* to X-ray.

As alcohols are said to act as depressive narcotic agents, several have been tried to see if gastrulation could be permanently suppressed by them without interfering markedly with subsequent growth and differentiation. While this was not found to be the case, except in instances of fatal injury to the more susceptible individuals, certain effects were observed which are described below. Blastula and young gastrula stages were exposed for about eighteen hours to various concentrations of methyl, ethyl, propyl, isopropyl, butyl, amyl and capryl alcohols. Since individuals of a culture vary widely in their susceptibility to the alcohols, the results are based on the condition of approximately fifty per cent or more of the embryos as determined by the study of at least three random samples from each culture.

In general alcohols act as depressive narcotic agents although they may exhibit exactly opposite effects in different concentrations (Lillie, 1912*a*). In very low concentrations they may stimulate development (Lillie, 1912*a*; Mast and Ibara, 1922, on tadpoles; Elhardt, 1930, on chick) while the higher concentrations are toxic. The higher alcohols are more toxic than the lower ones. Alcohol produces cyclopean eyes in *Fundulus* and also tends to suppress the development and differentiation of the auditory vesicles (Stockard, 1910). Certain concentrations will cause complete but reversible arrest of cleavage (Lillie, 1914). Eggs show a rhythmical susceptibility. The action of alcohol, and other anæsthetics, has been variously interpreted (Lillie, 1916; Henderson, 1930; Winterstein, 1926).

II

Reference to Tables I and II will show the approximate concentrations which produced certain effects. The concentrations are expressed as cubic centimeters of the alcohol which were added to 100 cc. of sea water containing the embryos. It will be noted that very little if any significant difference exists between the results for the two stages, indicating that they are probably equally sensitive. To avoid repetition only the results secured from the exposure to methyl alcohol will be described. The data for the others are included in the tables.

TABLE I

Influence of alcohols upon gastrulation and subsequent development during eighteen hours of exposure. Blastula stage.

Alcohol	Concentration of alcohol expressed in cc. per 100 cc. of sea water						
	Toleration. No effect on development	Retardation and inhibitory effects	Gastrulation only	Approximate limits of complete but reversible inhibition of gastrulation in some or all individuals	Complete inhibition of any gastrulation	All dead	Simple exogastrulæ which did not differentiate
Methyl	0.2–1.5	1.5–3.5	4 –5.5	5.0–5.9	5.5	6.0	2.5
Ethyl	0.5–0.9	1.0–2.0	2.0–3.6	2.7–4.4	3.8	5.0	0.9–1.3
Propyl	0.1–0.5	0.5–1.0	0.6–1.3	1.4–2.0	1.8	2.0	0.2–0.8
Isopropyl	0.1–1.5	1.6–2.0	2.0–2.4	2.1–3.1	3.2	3.5	1.0–1.7
Butyl	0.1–0.2	0.3	0.7	0.7–1.3	1.0	1.6	0.3–0.4
Amyl	0.1	0.1–0.2	0.3–0.4	0.4–0.6	0.5	0.7	0.1–0.3
Capryl		0.01	0.01–0.02	0.02–0.05	0.04	0.06	0.01–0.02

Methyl alcohol.—The embryos tolerate long exposure to concentrations from 0.2 cc.–1.5 cc. with no appreciable effect on gastrulation and development. In a 2 cc. concentration, development is slightly retarded but plutei develop. In a 2.5 cc. concentration, gastrulation and subsequent development are further retarded, and after 18 hours fewer top-swimming individuals are present. These are small plutei with frequent abnormal skeletal structures and inhibited arm development. A few undifferentiated cases show slight external outpushing of the vegetal pole cells or rupture in this region. Movement is slower.

In a concentration of 3 cc. gastrulation is further retarded or even inhibited and fewer stunted young plutei (especially with reference to arm development) are lethargically swimming through the medium. Most of the culture is on the bottom of the container. Individuals in which the vegetal pole cells are unevenly extruded or in which rupture, followed by some disintegration of the vegetal pole region, has occurred are quite common. The ectoderm of the embryos is ragged but the apical plate is present, even in embryos which do not go beyond the late gastrula stage. In the same length of time the control formed medium-sized plutei.

TABLE II

Influence of alcohols upon gastrulation during eighteen hours of exposure

Alcohol	Concentration of alcohol expressed in cc. per 100 cc. of sea water			
	Approximate limits of complete but reversible inhibition of further gastrulation in some or all individuals	All dead	Simple exogastrulæ which did not differentiate	Complete inhibition of any gastrulation
	Young Gastrula			
Methyl	3.9–5.5	5.9	0.4–2.5	5.2
Ethyl	2.0–4.0	4.7	0.5–1.5	3.5
Propyl	1.6–1.9	2.1	0.5–1.5	1.8
Isopropyl	2.2–2.6	3.0	0.3–1.4	2.3
Butyl	0.7–0.9	1.3	0.1–0.7	1.0
Amyl	0.4–0.6	0.7	0.1–0.2	0.5
Capryl	0.02–0.03	0.05	none	0.04

In a 3.5 cc. concentration the embryos develop as far as the triangular stage with differentiation of the apical plate, but lack skeleton. In a 4 cc. concentration the embryos only go as far as the late gastrula in 18 hours. A 4.5 cc. concentration gives shallow or early gastrulæ and their movement is very slow and tends to be rotational, while 5 cc. gives only shallow gastrulæ at the most, and many blastulæ.

Gastrulation is inhibited for the most part at a concentration of 5.5 cc. although a few show initial gastrulation. There is very little movement. On return to fresh sea water after 18 hours in the solution, these blastulæ gastrulate and continue development. In concentrations from 5.6 cc.–5.9 cc. fewer and fewer blastulæ survive and these gastrulate in fresh sea water, although frequently in an abnormal manner. Subsequent differentiation is abnormal and many do not go beyond a late gastrula stage. In a concentration of 6 cc. the embryos do not survive the experimental period.

Above a minimal concentration of the alcohols the general effect is a depression of gastrulation and subsequent development which is proportional to the kind of alcohol, the concentration and the length of exposure. Methyl is least depressive and capryl the most, while isopropyl is less depressive than the normal alcohol. Lillie (1914) found the latter to be the case in his study of the action of alcohols in suppressing cleavage in sea-urchin eggs. The different alcohols give quite similar results but within progressively narrower limits for the higher ones. The present series was found by Pantin (1930) to hold for the action of alcohols on the ameboid movement of *Amœba* and by Lillie (1914) in the suppression of cleavage.

Gastrulation may be inhibited by alcohol for at least as long as 18 hours without loss on the part of some embryos of the ability to gastrulate and without appreciably detrimental effect on whatever processes are involved. When returned to fresh sea water, the surviving blastulæ gastrulate with apparently undiminished vigor and subsequent development may be quite typical. When developmental abnormalities appear, they are associated with differentiation, i.e., formation of the skeleton, size of the body, and position and length of the arms. These plutei are less resistant and die sooner than do the control. This temporary inhibition of gastrulation is shown by the following observations of embryos exposed to ethyl alcohol which is only slightly more toxic than methyl.

After 5 hours in a 3 cc. concentration the embryos were still in the oval blastula stage. Lethargic movement was visible but all were on the bottom of the container. At this time the controls were at the late gastrula or triangular gastrula. When changed to fresh sea water these developed after 17 hours to the late triangular and early pluteus stages. A few, consisting of the weaker individuals, were at the blastula stage or showed irregular gastrulation with some slight external development of the vegetal pole cells or rupture of the vegetal pole region. After the same length of time in a 5 cc. concentration, no movement was visible and there was considerable evidence of cellular swelling and disintegration especially at the vegetal pole region. Seventeen hours after changing to fresh sea water the embryos were at the late triangular gastrula stage with large skeletal spicules. At this time the control was at the young pluteus stage. Twenty-four hours later the embryos had developed into normally appearing individuals with no skeletal peculiarities as far as were seen. A 5 cc. concentration was fatal during an exposure of 18 hours.

If inhibition occurs during gastrulation, it is resumed at the point where it left off and goes on to completion with the removal of the alcohol. During the interim nothing vital to the process has been removed, passed over or destroyed. Under the influence of the alcohol the stage of gastrulation does not express itself as determining the capacity to continue gastrulation in the modified environment. Concentrations which inhibit gastrulation at a late cleavage or blastula stage likewise stop it after it has begun or during the process. Thus no difference in sensitivity was demonstrated. Only death or fatal injury completely inhibited typical gastrulation as tested by the ability of the embryos to undergo further development.

Gastrulation is not apparently more susceptible to an anæsthetic like alcohol than other developmental processes. Differentiation without gastrulation was not seen, nor gastrulation without differentiation except in fatally injured embryos. Such embryos live for only a short time after transfer to fresh sea water and gastrulation is not typical. These may show an irregular proliferation of cells at the vegetal pole or the blastocœl becomes packed with cells. The few cases of apparent initial exogastrulation which appeared in lower concentrations and after shorter exposure periods were of this type or showed a very shallow or irregular outpushing of the vegetal pole cells. Only some of the more susceptible individuals in the lower concentrations showed this condition. They did not differentiate beyond forming an apical plate and a small, triangular body form. Frequently the blastocœl was more or less packed with cells.

Abnormal embryos, no matter how produced, always tend to swim in a small circular path. The more retarded or abnormal the structure, the shorter is the diameter of the circle. Modification of shape would account for this in part, but, where embryos are very retarded though otherwise quite symmetrical, there does occur a modification of ciliary distribution and size. The cilia of the blastula and gastrula stages are least sensitive to the narcotic action of alcohol. In concentrations which inhibited development beyond the stage at which it was put into the solution, the cilia continued to beat but at a slower rate. In concentrations immediately above this value, movement was much reduced but it was stopped completely only with complete death or disintegration of the embryo. In lower concentrations, the rate is unaffected but becomes gradually slower above a minimal concentration. The change does not parallel developmental inhibition. This difference in the narcotizing concentration for different tissues is well known. Lillie (1912*a*) found that the cilia of *Arenicola* larvæ continued their activity for hours in isotonic sugar or magnesium chloride solutions, ether and chloroform, which completely inhibited any muscular movement. In the present study the coördination of the ciliary action was not apparently disturbed although the pathways of the embryos tended towards small circles. Dying embryos did show a fluttering effect attributable to disintegration.

One of the first of the differentiating tissues of the embryo to be affected by the treatment with alcohol is the mesenchyme and this becomes apparent in the modification of the skeleton. This effect has been observed by Runnström (1928) and others (Waterman, 1932, 1934) following the use of LiCl, various other salts, and also physical agents. The modifications first appear in concentrations just above the limit of toleration and are evident in the irregularity and loss of symmetry of the skeletal parts. The ends of the body rods are smooth and unfused. In higher concentrations there appear suppression and reduction of the skeletal rods on one side or both, difference in thickness and amount, accessory parts or projections, and abnormal positions. Alcohol thus markedly affects the distribution and activity of the skeleton-forming mesenchyme, even when the remainder of the embryo may be quite typical in general appearance for the particular stage.

Another modification is that of the fusion of embryos. This varied from pairs up to large aggregations. Only those fusions involving two embryos underwent any considerable differentiation although the members of a large aggregation might gastrulate. The former are comparable to those secured by other means and by other investigators (cf. Waterman, 1932, 1934). Fusion seemed to occur at regions of initial disintegration, and, since this occurred generally in the region of the vegetal pole, many cases failed to gastrulate or did so in a very irregular manner. This region is more susceptible and is the one which seems to be most active physiologically and developmentally at this time (Child, 1928). The swelling and partial separation of the blastomeres in this region point to an injurious permeability change. The final result is an irregular mass of swollen, spherical cells which gradually disintegrates.

That weak solutions of the alcohols may give a slight acceleration of development seems to have been found in the course of this study. This was seen to occur with methyl, ethyl and isopropyl alcohols in the lowest concentrations employed. In the case of the earliest stage the embryos for the most part gastrulated sooner, and in all cases the plutei matured more quickly than the control. They did not survive as long as the control. There was, however, no noticeable difference in final size. Others have likewise found that weak solutions of certain alcohols accelerate development. In a study of the effect of ethyl alcohol on tadpoles, Mast and Ibara (1922) found that, although there was a reduction in activity of the tadpoles, there occurred an increase in the rate of growth and a decrease in the rate of mortality. While it is not clear as to how these effects were produced, they suggest that the reduction in activity might account for the longer life and that the alcohol served as food which conserved the reserve food material in the tadpole's body. The animals were not fed during the experiment. Alcohol may also increase irritability when used in low concentrations, while exhibiting typical anæsthetic action in higher concentrations. Reference should be made to Lillie (1912*a*), page 374, for a discussion of this point and additional examples. More recently Elhardt (1930) has found that young chicks respond by an increased growth rate to doses of ethyl alcohol. Methyl, propyl and butyl alcohols (1932) had a stimulating effect on growth for a time.

SUMMARY

The general effect of immersing the blastula and young gastrula stages in various concentrations of methyl, ethyl, isopropyl, propyl, butyl, amyl and capryl alcohols for 5–18 hours is a depression of gastrulation and development which is conditioned by the kind of alcohol, the concentration and the length of exposure. They all give quite similar effects but within narrower limits for the higher ones.

Apparently the process of gastrulation is no more sensitive to the alcohols than other developmental processes. It may be inhibited for 5–18 hours without the ability or vigor of gastrulation being diminished. The stage of gastrulation does not express itself as determining the capacity to continue gastrulation in this modified environment. Differentiation without gastrulation was not found nor gastrulation without differentiation except in fatally injured embryos. Information is given on the concentrations which caused complete but reversible inhibition of gastrulation and on the limits of alcohol toleration. A slight acceleration of the rate of development was observed in the lowest concentrations of methyl, ethyl and isopropyl alcohols.

Embryonic differentiation is affected by the alcohols. Abnormalities appeared in the development of body form and size, length of the arms, and structure and amount of the skeleton. Ciliary activity is

not inhibited in concentrations which stop development but the rate is reduced. Fusions appeared in the cultures but only those involving two individuals underwent any considerable differentiation. Few exogastrulæ were found and they were always of a shallow type which did not differentiate to any extent. This abnormality appeared among the more susceptible individuals in the lower concentrations of the alcohols.

LITERATURE CITED

Baldwin, F. M., 1920. Susceptible and resistant phases of the dividing sea-urchin egg when subjected to various concentrations of lipoid-soluble substances, especially the higher alcohols. *Biol. Bull.*, **38**: 123.

Child, C. M., 1928. The physiological gradients. *Protoplasma*, **5**: 447.

Curtis, W. C., J. A. Cameron and K. O. Mills, 1936. Exogastrulation in Amphibia after X-ray exposure. *Science*, **83**: 354.

Elhardt, W. E., 1930. Effect of ethyl alcohol on the growth of chicks. *Am. Jour. Physiol.*, **92**: 450.

Henderson, V. E., 1930. The present status of the theories of narcosis. *Physiol. Rev.*, **10**: 171.

Holtfreter, J., 1933. Die Totale Exogastrulation, eine Selbstablösung des Ektoderms vom Entomesoderm. *Arch. f. Entw.-mech. d. Organismen.*, **129**: 669.

Lillie, R. S., 1912*a*. Antagonism between salts and anæsthetics. I. *Am. Jour. Physiol.*, **29**: 372.

Lillie, R. S., 1912*b*. Antagonism between salts and anæsthetics. II. *Am. Jour. Physiol.*, **30**: 1.

Lillie, R. S., 1914. The action of various anæsthetics in suppressing cell-division in sea-urchin eggs. *Jour. Biol. Chem.*, **17**: 121.

Lillie, R. S., 1916. The theory of anæsthesia. *Biol. Bull.*, **30**: 311.

Lillie, R. S., 1918. The increase of permeability to water in fertilized sea-urchin eggs and the influence of cyanide and anæsthetics upon this change. *Am. Jour. Physiol.*, **45**: 406.

Mast, S. O., and Y. Ibara, 1922. Effect of ethyl alcohol on tadpoles. *Am. Jour. Physiol.*, **59**: 294.

Pantin, C. F. A., 1930. On the physiology of amœboid movement. VII. The action of anæsthetics. *Proc. Roy. Soc. London, B* **105**: 565.

Runnström, J., 1928. Zur experimentellen Analyse der Wirkung des Lithiums auf den Seeigelkeim. *Acta Zoölogica*, **9**: 365.

Stockard, C. R., 1910. The influence of alcohol and other anæsthetics on embryonic development. *Am. Jour. Anat.*, **10**: 369.

Waterman, A. J., 1932. Effects of isotonic salt solutions upon the development of the blastula of the sea-urchin Paracentrotus lividus. *Arch. de Biol.*, **43**: 471.

Waterman, A. J., 1934. Further studies of exogastrulation in the sea urchin. *Biol. Bull.*, **67**: 172.

Winterstein, H., 1926. Die Narkose. Second Edition. Julius Springer, Berlin.

REGENERATION IN MNEMIOPSIS LEIDYI, AGASSIZ[1]

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The only report giving results of experiments dealing with regeneration in ctenophores has been made by Mortensen (1913). His experiments, which were done on only four specimens of *Bolina infundibulum*, served to demonstrate that these animals possess the power to regenerate lost parts. These experiments of Mortensen, as well as my observations of regenerating specimens made incidentally while collecting *Mnemiopsis*, disprove the common conception that ctenophores will not regenerate. These observations led to experiments which deal with the regeneration of organs and other parts that have been removed from the body of a ctenophore, *Mnemiopsis*. It is the purpose of this report to present these experiments. Further investigation which may give solution in part to problems depending on some aspect of regeneration in this animal will be reported at a later date.

METHODS

The specimens were measured along the three main axes of the body and cut into parts with scissors. Each piece was kept in a separate finger bowl during the period of regeneration. The finger bowls were partly immersed in running water in order to maintain a fairly low and constant temperature. The temperature remained near 21° C. during most of the period of observation, reaching a low of 18.9° C. and a high of 23° C. This range of temperature was satisfactory since the survival of the regenerating pieces was high. These pieces were observed through a dissecting microscope at frequent intervals to note the progress of regeneration.

All of the photographs shown in this report were taken with a Leica camera set up with an extension tube outfit. A black background was placed beneath the specimens and out of the focal range of the lens. The lighting consisted of one Spencer microscope lamp placed in a horizontal position and one photoflood lamp placed above the specimens. These animals were photographed while in a large finger bowl filled with a solution of sea water and a low percentage of alcohol. The alcohol stopped the movements of the animal except those of the paddle plates. The rate of movement of these plates was decreased sufficiently to allow time exposures.

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[1] Contribution No. 16 from the Department of Biology, Brooklyn College.

EXPERIMENTS

The sequence of recognizable phases during regeneration in *Mnemiopsis* was wound closure, swelling and stretching, and reformation of lost parts. Wound closure was accomplished by the edges of the outer body wall moving together and fusing at the cut area. At this time the corresponding canals and rows of plates fused with each other. Wound closure was complete five hours after the operation. The swelling and stretching of the region at the regenerating area was indicated by the stretching of the rows of plates (Fig. 1). Stretching was complete within 18 hours and at this time the plates on these rows had become widely separated (Fig. 1). Plates regenerated on the rows at regular intervals between the old plates within a few hours. When cuts were made to remove parts of the body containing rows of plates or the apical organ, these structures were reformed. In the sequence of regeneration of organs which had been removed, the apical organ formed first and the regeneration of rows of plates followed within a few hours. Plates regenerated on these rows within three hours after the rows were complete. The experiments reported here are grouped according to the following headings: (1) regenerating halves; (2) regenerating thirds; (3) regenerating fourths and eighths.

Regenerating Halves

Specimens were divided into halves by a cut across the body midway between the apical and oral ends, by a longitudinal cut midway between the adesophageal plate rows (*a–b*, Fig. *A*), and by a longitudinal cut midway between the adtentacular plate rows (*c–d*, Fig. *A*).

Regeneration following the cutting across between the apical and oral ends gave the following results. New plates were formed between the old ones along the stretched rows at the oral end of the apical pieces within five days. Additional plates were formed also at the apical end of the oral pieces within three days. An apical organ was formed in most of the oral pieces within two days.

The specimens which were divided into halves along the long axis of the body between the adesophageal plate rows were cut at the midline except in the region of the apical organ. In this region the cut was made just to one side of the apical organ in order to leave this organ in one of the halves. Fifteen of the 20 halves which retained the apical organ lived and each regenerated 4 complete rows of plates within 9 days. Two views of one of these regenerating halves are shown in Figs. 2 and 3. Of the 13 halves which lived and which did not retain the original apical organ, 3 reformed this organ and 4 complete rows of plates within 11 days, 4 rounded up and formed the apical organ without regenerating any rows of plates, and 6 showed no signs of regenerating either an apical organ or rows of plates by the end of the tenth day. The halves which formed the apical organ without regenerating any rows of plates rounded up and behaved as normal specimens.

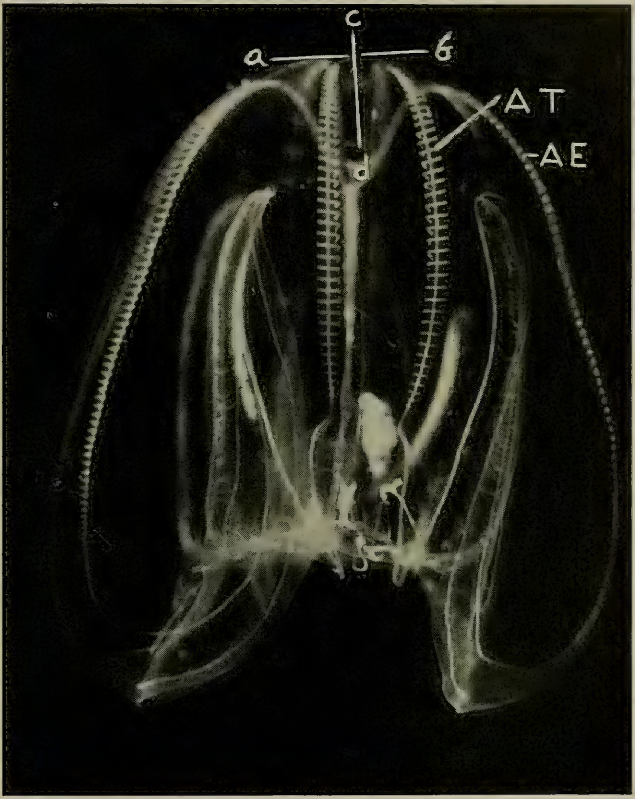

FIG. *A*. This is a photograph of a living animal. It shows some of the internal organs and above it are lines to show planes through which cuts were made. This specimen was fed some clam juice and this food caused the internal organs to show. Some of this food can be seen near the mouth of this specimen. Lines *a–b* and *c–d* show the adesophageal and the adtentacular planes respectively. *AE*, adesophageal row, and *AT*, adtentacular row.

The animals which were divided into halves along the long axis of the body between the adtentacular plate rows were cut at the midline except in the apical region. In this region the cut was made to one side of the apical organ in order to leave this structure in one of the halves. Eleven of the 20 pieces which retained the apical organ lived and each regenerated 4 complete rows of plates within 8 days. Only 5 of the 20 pieces which did not retain the original apical organ lived and of these one formed this organ and 4 complete rows of plates while the remaining 4 specimens failed to regenerate either an apical organ or rows of plates.

Regenerating Thirds

In this experiment the animals were divided into thirds by two cross cuts between the apical and oral ends of the body. By these cuts each specimen was divided into an apical piece, a middle piece, and an oral piece. Each of the 15 apical pieces survived and formed the row and canal connections as well as lobes and auricles at the oral end. Twelve of 15 mid-pieces lived and regenerated the apical organ with the regular canal connections in the apical zone (Fig. 1), and canal connections as well as lobes and auricles in the oral end. Each of the 13 oral surviving pieces regenerated an apical organ and formed the regular canal connections. Regeneration was complete in all apical, middle, and oral pieces within 7 days.

Regenerating Fourths and Eighths

Specimens were divided into fourths by three methods of cutting. According to one method two cuts were made at right angles to each other, one between the adesophageal plate rows and the other between

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EXPLANATION OF PLATE I

All photographs in this plate were made of living animals. *A*, apical organ; *AU*, auricle; *M*, mouth region; *P*, plate; *PR*, plate row; and *S*, stomodeum.

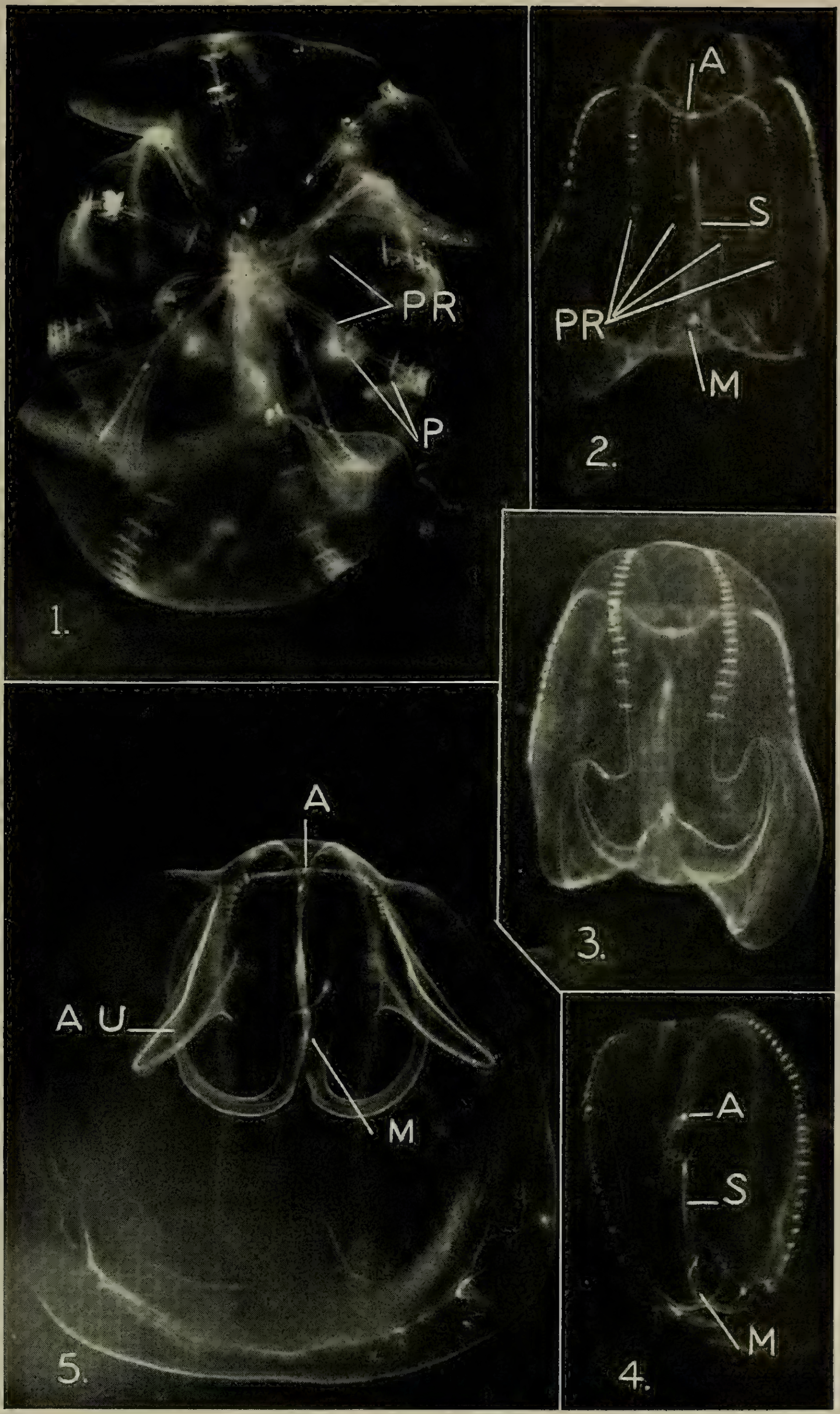
FIG. 1. This shows the apical end of a regenerating mid-piece of an animal which had been cut into thirds. The stretched rows are shown at *PR* with the separated plates at *P*. Later additional plates were formed on these rows between the old plates.

FIG. 2. This shows a regenerating half of a specimen which had been cut along the long axis of the body between the adesophageal rows. The regenerated rows of plates can be seen at *PR*.

FIG. 3. This is a view of the normal surface of the animal half shown in Fig. 2. The old rows can be seen clearly.

FIG. 4. This is a photograph of an apical quarter of a specimen showing the regenerating surface. This piece was produced by cutting a specimen across between the apical and oral ends and by another cut between the adtentacular rows. The piece shown here retained the original apical organ. Note the regenerating rows of plates.

FIG. 5. This is a photograph of an oral quarter of a specimen which had been cut as the one shown in Fig. 4. This piece did not regenerate any rows. It rounded up after regenerating an apical organ and behaved as a normal animal.


PR
P
1.
A
S
PR
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A U
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5.
A
S
M
4.

PLATE I

the adtentacular plate rows, through the longitudinal axis of the body. By another method one cut was made across the animal midway between the apical and oral ends and each of these halves was cut longitudinally between the adesophageal plate rows. The third method was similar to the second one except that the longitudinal cut was made between the adtentacular plate rows.

The following is the result of regeneration in the pieces produced by the first method of cutting. Sixty-five of 80 pieces survived and 5 of these formed 6 rows of plates and an apical organ each within 7 days. Of the 32 pieces which failed to regenerate by the eleventh day only 3 had retained the original apical organ. Each of the remaining 28 pieces formed from one to four plate rows within 7 days. The experiment was discontinued on the eleventh day and at this time each of the 28 pieces showed definite signs of regenerating completely the parts which had been removed by the operation.

Four of the 14 fourths retaining the original apical organ which were produced by the second method of cutting formed 4 rows of plates within 7 days. Seven of the 14 pieces healed and failed to regenerate any rows of plates but rounded up and behaved as normal animals. The remaining 3 pieces failed to round up or to regenerate any rows of plates. Four of the 12 apical pieces which did not retain the original apical organ formed this organ with canal connections within 7 days. Plate rows were not formed during this time and each of the specimens reacted as normal animals. One of the apical pieces regenerated both an apical organ and 4 rows of plates within 7 days. Each of the remaining 12 pieces failed to regenerate either plate rows or an apical organ and when they were discarded at the end of the seventh day they showed no evidence of regeneration. Of the 22 oral fourths, 11 regenerated the apical organ and rounded up to form individuals which behaved as normal animals though with only the 4 original rows of plates. All of the remaining oral pieces failed to regenerate either the apical organ or the rows of plates.

All of the 15 apical quarters produced by the third method of cutting and which retained the apical organ survived and 6 of them regenerated 4 complete rows of plates (Fig. 4) while each of the remaining 9 pieces rounded up without regenerating any of the lost rows of plates and behaved as a normal animal. Only 9 of the 15 apical pieces which did not retain the apical organ lived and 8 of these formed this organ, rounded up and continued to live without further regeneration. The remaining one of the apical pieces regenerated both the apical organ and 4 rows of plates. Twenty-seven of the 30 oral pieces survived and of these only one regenerated both the apical organ and 4 rows of plates, 18 regenerated only the apical organ and rounded up (Fig. 5), while 8 failed to regenerate either the apical organ or any rows of plates.

Eighteen specimens were cut into fourths by cuts passing through the adesophageal and the adtentacular planes. Each fourth was then cut across midway between its apical and oral ends to produce eighths. All of these eighths died within 28 hours and at this time had given no evidence of regenerating.

DISCUSSION

The results of experiments whereby various parts of the body of *Mnemiopsis* were reformed show conclusively that these animals will regenerate the lost parts. This in itself is significant since ctenophores have been regarded as a group without the power of regeneration. The rapidity of regeneration in *Mnemiopsis* and the transparency of its body which makes observations clear and convenient are also important features possessed by this animal. Experiments on *Mnemiopsis* show further that this animal possesses a regulating center. The halves and fourths produced by longitudinal cuts which retained the apical organ regenerated the rows of plates within less time and in a higher percentage of cases than those pieces which did not retain this organ. Furthermore, in all portions of *Mnemiopsis* which did not retain the original apical organ this structure preceded the rows of plates in regeneration in all cases. I therefore conclude that the apical organ in *Mnemiopsis* is the regulating center during regeneration. The apical organ previously has been considered (Coonfield, 1936) as a region of dominance and the conclusion expressed in this report gives further proof that this organ constitutes an important center in this animal.

It is interesting to note that following longitudinal cutting in *Mnemiopsis* certain pieces failed to form the lost rows of plates, rounded up and continued to live as normal animals. Some of these pieces had retained the original apical organ while others were without this organ after the cutting. Those pieces which did not retain the original apical organ soon regenerated this structure. It is clear then that all of these pieces which rounded up were unable to carry out reconstitution to completion.

CONCLUSIONS

1. Halves, thirds, and fourths of *Mnemiopsis* regenerated the lost parts while eighths of this animal failed to regenerate.

2. Some of the halves and fourths failed to regenerate rows of plates and these pieces continued to live as normal animals.

3. The sequence of regeneration of organs was the apical organ, rows of plates, and plates on these rows. The plates were complete within three days after the formation of the rows.

4. Certain evidences brought out by regenerating pieces of *Mnemiopsis* show that the apical organ of this animal is the regulating center.

LITERATURE CITED

Coonfield, B. R., 1936. Apical dominance and polarity in Mnemiopsis leidyi, Agassiz. *Biol. Bull.*, **70**: 460.

Mortensen, Th., 1913. On regeneration in ctenophores. *Vidensk. Meddel. fra Dansk naturh. Foren.*, **66**: 45.

SENSORY HAIRS OF THE DOGFISH EAR

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Sensory hairs from the crista of the fresh water teleost, *Ameiurus nebulosus*, have been viewed in living condition and observations have been recorded on the nature and distribution of these hairs (Bowen, 1932). The conclusions reached through the study of such living materials are different in some significant features from those which have been arrived at, by different investigators, from examination of preserved ampullæ.

The internal ears of elasmobranchs have been the subject of numerous investigations and the general structure of those in the dogfish is well known (Retzius, 1881). Due to their comparatively large size and ready accessibility, the internal ears of dogfish have been studied repeatedly in relation to equilibrium (Maxwell, 1923). Examination of the sensory endings in the labyrinth, and particularly the sensory hairs of the crista in elasmobranchs, however, has been limited to preserved materials.

In order to determine more accurately the nature of the ampullar organs in the internal ear of elasmobranchs, observations have been made of fresh ampullæ from the dogfish, dissections for these studies being made according to the technique which has been described for *Ameiurus*. This paper deals with the sensory hairs of the cristæ as they appear in living condition in their natural medium, endolymph, and in media differing somewhat in composition from such endolymph.

These observations were carried on for the most part in the Marine Biological Laboratory, Woods Hole, Massachusetts. Records of the examinations were secured with photomicrographic apparatus made available through the generosity of the Elizabeth Thompson Science Fund.

In order to determine with some precision the concentration of fluid necessary for maintaining the hair cells of the dogfish ear as in the intact animal, it was found necessary to examine cristæ in different media and to note the effects of each upon the sensory hairs. Cristæ, therefore, were examined in the following solutions: Ringer's solution of varying concentrations; different dilutions of sea water; distilled water; sea water itself; sea water to which additional sodium chloride had been added.

Initial dissections of the crista, for which *Mustelus canis* and *Squalus acanthias* were employed, were made in Ringer's solution and in dilutions of sea water approximating Ringer's solution in concentration (United States Bureau of Fisheries Laboratory and Harvard Zoölogical Laboratory). Similar dissections of cristæ from the ampullæ of four sharks of the species *Carcharius commersonii* were made at the Bermuda Biological Station. These preparations, dissected in media of saline content less than sea water, consistently failed to show sensory hairs in any condition approximating those found in *Ameiurus*. In most of these cristæ no sensory hairs whatever could be found. In others there were bare traces of the projecting hairs, these being reduced to a small fraction of the length of sensory hairs such as are found in *Ameiurus*.

Through repeated trials with numerous later dissections it has been found that the sensory hairs of *Mustelus canis* maintain their size and appearance to best advantage when they are kept bathed in sea water and not in other solutions of higher or lower concentrations. Deviation from the concentration furnished by sea water tends to produce decided alterations in the sensory hairs. In solutions slightly lower or slightly higher in saline content there is a reduction in the number of hairs and with greater deviation a further reduction in size and number of these structures. Fresh dissections in sea water, however, invariably show the sensory hairs in good condition.

The sensory hairs, when viewed in cristæ removed from the ampullæ in sea water and held in such medium for observation under the higher magnifications, are found to be slender, tapering processes which extend at right angles from the sensory surface. The sensory hairs of the dogfish range between 35 μ and 60 μ in length, the latter figure representing the length of the greater number of the undisturbed processes. In *Ameiurus* the length of the sensory hairs is near 50 μ. The slight deviation of the hairs, near the middle of the crista, from the midpoint toward either end, a structural feature which was recorded for *Ameiurus*, is not noted in the dogfish.

The sensory hairs of the dogfish crista are, in some instances, maintained without appreciable alteration for a matter of hours in sea water. After three or four hours degenerative changes may appear and, in some preparations, the hairs have been seen to shorten and become reduced in number. Complete disappearance occurs some hours later.

In preparations which have been kept for approximately an hour in sea water or for a shorter time in solutions of slightly greater or less saline content than sea water, protrusions of clear, mucus-like material form along the surface of the sensory layer. In a short time this substance increases in amount and globules of the material, becoming entirely detached from the neuroepithelial surface, remain near the bases of the sensory hairs. This material seems to be rather of the nature of a secretion than a product of cytolysis, for if, in the early stages of its formation and before the destruction of the hairs occurs, the crista is washed in sea water, the globules are removed though the sensory hairs remain. It has not yet been found possible to determine definitely whether these materials arise from the sensory cells or from the supporting cells. The position of the globules, when they first appear near the bases of the sensory hairs, suggests that the materials are coming from the supporting cells.

The hairs in one preparation were found to be moving. In this case the crista was first observed under high magnification about half an hour after the decapitation of the fish. Sensory hairs were moving like cilia when the crista was first seen and continued in motion for about thirty minutes from the time of the first observation. This fact in itself indicates that the hair, in sea water, is in a medium which does not immediately cause marked injury to it.

It has recently been found possible in two cases to secure views of the sensory hairs of the crista without removing the ampulla from its position in the chondrocranium and with comparatively little injury to the fish. In these dissections a small opening was cut in the cartilaginous capsule immediately above the left anterior ampulla and the perilymph drawn off with a fine pipette. A slit was then made in the roof of the ampulla immediately above the crista and the walls of the ampulla held away from the field of vision by means of fine glass needles. The sensory hairs could be seen, somewhat dimly it is true, but nevertheless recognizable, through the use of vertical illumination. The cristæ in these two cases were in living animals, in endolymph relatively undisturbed and with the eighth nerve intact. Though optical conditions were not suitable for detailed examination, enough could here be determined as to size, shape and arrangement of the sensory hairs to indicate that the preparations in sea water closely approximated the normal.

The cristæ of the dogfish ear, though similar to those of *Ameiurus*, show some differences, the significance of which must await further investigation. In *Ameiurus* the crista extends slightly farther into the ampulla and the sensory layer forms more nearly a straight line across the lumen. Whether this more complete blocking of the lumen in *Ameiurus* is of functional significance is not known. The position of the hairs extending directly outward from the sensory layer both in the dogfish and in *Ameiurus* is contrary to the usual description and is a factor which must be considered in any final explanation of the rôle of the crista in equilibrium.

Likewise, the movement of the sensory hairs of the cristæ (Bowen, 1931, 1932, 1935) may have some definite function in the physiology of the labyrinth. What that function may be is still far from evident. The movement of cilia within the cavities of the internal ear of *Petromyzon*, first mentioned by Ecker (1844) has been nicely demonstrated by de Burlet and Versteegh (1930) and the structure of the ear given in much detail. The cilia, found by these investigators to be moving in *Petromyzon*, appear distinct from the sensory hairs of the cristæ. Whether there is any structural or functional relationship between the ciliated epithelium of the cavities and the sensory hairs of the cristæ in *Petromyzon*, and whether the latter are ever in motion are questions not yet answered.

Finally, the formation and appearance of the minute globules, which have been seen to arise among the bases of the sensory hairs in the dogfish ear suggest that material so formed may be a secretion. These globules apparently do not represent disintegration products since they can be washed away, after which the tissues which have been covered with them remain normal. Though the present evidence is indicative only, it is quite possible that this material, arising as a secretion from the neuroepithelium, goes to make up a part, if not all of the cupular substance, a substance which under the influence of fixing reagents forms the cupula of common description.

REFERENCES

BOWEN, R. E., 1931. Movement of the so-called hairs in the ampullar organs of fish ears. *Proc. Nat. Acad. Sci.*, **17**: 192.

BOWEN, R. E., 1932. The ampullar organs of the ear. *Jour. Comp. Neur.*, **55**: 273.

BOWEN, R. E., 1935. Movement of sensory hairs in the ear. *Proc. Nat. Acad. Sci.*, **21**: 233.

BURLET, H. M. DE and C. VERSTEEGH, 1930. Ueber Bau und Funktion des Petromyzonlabyrinthes. *Acta Oto-Laryngologica*, Supplement XIII.

ECKER, A., 1844. Flimmerbewegung im Gehörorgan von Petromyzon marinus. *Arch. Anat. Physiol.*, p. 520.

MAXWELL, S. S., 1923. Labyrinth and Equilibrium. Philadelphia.

RETZIUS, G., 1881. Das Gehörorgan der Wirbelthiere. I. Das Gehörorgan der Fische unde Amphibien. Das Gehörorgan von Acanthias vulgaris, pp. 109–117, Tafel XVIII und XIX, Stockholm.

. THE MORPHOLOGY AND BEHAVIOR OF THE INTRACELLULAR BACTEROIDS OF ROACHES[1]

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The intracellular bacteroids of roaches, some ants, beetles, and other invertebrates, as well as the intracellular yeast-like bodies of aphids, coccids and other arthropods, have been the subject of much work and speculation, ably reviewed by Buchner (1921, 1930) and Glaser (1930). Their morphology and parts of their life cycles have been studied by Mansour (1934), Lilienstern (1932), Koch (1931), Meyer (1925), Florence (1924), Buchner (1912, 1923) and others, but in many of these studies critical points have been passed over because of insufficient material or technical difficulties. It has been definitely established, however, that these intracellular bodies occur in all individuals of the host species and are regularly transmitted to the offspring.

Most workers in the field agree that these bodies are bacteria in some cases and yeasts in others, but some biologists yet prefer to interpret them as mitochondria or waste products as did Cuénot (1896) and Henneguy (1904). Cultivation of the bacteroids on artificial media has been attempted (Mercier, 1907; Hertig, 1921; Glaser, 1930; and others) with negative or questionable results in most cases. My own work on cultivation, which to date is negative, will be published later. If these bacteroids can be cultivated outside the invertebrate host, they must be bacteria; if they cannot be cultivated, they may be bacteria of the same order as *Rickettsia,* they may be other very highly specialized bacteria, or they may be non-living inclusions. We can, however, put considerable reliance on studies of their morphology, staining reactions, and behavior.

The physiology of these intracellular bodies is entirely unknown. One group of workers (Buchner, Florence, Schwartz, and others) considers the relationship to be a true symbiosis between intracellular "symbiont" and invertebrate "host." Another group (Glaser, Mansour, and others) thinks of the relationship rather as being one of commensalism or mild parasitism. Neither faction, however, has advanced any substantial evidence for its contentions. In view of this situation it seems desirable to avoid such terms as "symbiont," "symbiosis," "parasite," and "parasitism" and the implications arising from their usage, and to designate the intracellular bodies in roaches merely as "bacteroids."

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Although the roaches were among the first insects found to harbor intracellular bacteroids (Blochmann, 1888) and there have been reports on various phases of their morphology, staining reactions, and life cycle, no detailed unified work on these bacteroids has been published. Heymons (1895) followed their behavior in a general way while studying the embryology of several species of roaches; Mercier (1907), Buchner (1912) and Fraenkel (1921) added materially to our knowledge of their morphology and life cycle in *Periplaneta* (*Blatta*) *orientalis;* Neukomm (1927) studied their morphology and staining reactions in *Blattella germanica;* and others have contributed details now and then. There is, however, little agreement among the workers and many points have been untouched. Concerning the present knowledge of the bacteroids of roaches, Glaser (1930*a*) wrote, "Many details in the chain of events are entirely lacking, and if known would undoubtedly assist much in the proper interpretation. . . . How non-motile elements are transported along definite, and in many cases, at least, rather sluggish, channels and find their way from the adult host cells to the immature ova and return is entirely unknown."

Materials and Methods

The work was concentrated on the large house roach, *Periplaneta americana,* collected at Pittsburg, Kansas, 400 embryos and 100 nymphs and adults being examined. Over 100 embryos and 80 nymphs of *Parcoblatta pennsylvanica,* collected at Pittsburg, Kansas, and Bloomington, Indiana, and smaller numbers of the following species were studied: *Parcoblatta uhlerania, P. latta, Blattella germanica,* and *Blatta orientalis,* collected at Bloomington and Winona Lake, Indiana; *Periplaneta australasiæ* and *Eurycotis floridana* from Dunedin, Florida; and *Cryptocercus punctulatus* from Knoxville, Tennessee. Many of the specimens were identified by Dr. J. A. Rehn, and the remainder by Dr. W. S. Blatchley.

The roaches were kept in the laboratory in closed metal or glass containers. Water was supplied either in a shallow dish or by wet filter paper. *Cryptocercus punctulatus* was fed filter paper and partially rotted pine wood; all others were fed potato, banana, and a little raw beef.

Eggs for embryological studies were obtained and cared for as follows. As soon as a female was observed to be ovipositing, she was placed in a separate container. If the oötheca was not dropped within 36 hours it was released by a gentle twist and the roach returned to the breeding pen. The oöthecæ were placed in Syracuse watch glasses in a larger container with a high relative humidity. The eggs of *Periplaneta americana* were incubated at 33° C.; others were left at room temperature. Under these conditions, the incubation time was approximately 30 days for *Periplaneta americana,* 36 days for *Parcoblatta pennsylvanica* and 40 days for *Blatta orientalis.* The oöthecæ of *Blattella germanica* were removed from the females at the time they were to be fixed.

Cytological methods were employed for studying the bacteroids in the gonads and fat tissues. Most of this material was fixed in Flemming or Champy, sectioned in paraffin at 5 μ, and stained in Haidenhain's iron hæmatoxylin. Other fixatives and stains were used as checks. Living material, with and without vital stains, was studied in considerable quantities.

Mature ovarian eggs were fixed in Flemming 12 to 18 hours or in Carnoy-Lebrun 10 to 12 hours and sectioned by the method described by Slifer and King (1933). These eggs were stained either with Giemsa (Wolbach, 1919), or better by Delafield's hæmatoxylin 10 to 15 minutes and destained until only the chromatin and possibly the yolk retained some color, followed by anilin safranin 12 to 20 hours and differentiated in 95 per cent alcohol. The latter method stains the chromatin black, bacteroids purplish red, and yolk pink. Other fixatives and stains were tried with varying success, but most fixatives make the yolk too brittle, and most stains have a greater affinity for the yolk than for the bacteroids.

The following method was adopted for fixing and staining embryos of all stages. The oötheca was opened slightly and dropped into Carnoy-Lebrun fixative. After two hours, the halves of the oötheca were separated, the unbroken chorions opened and the embryos returned to the fixative for a total of 20 to 24 hours. After washing with 95 per cent alcohol, each unbroken embryo was carefully removed from the oötheca and as much of the chorion teased off as possible. The embryos were soaked in 85 per cent alcohol containing 4 per cent phenol, dehydrated, embedded in paraffin, trimmed, and soaked in water 36 to 48 hours as recommended by Slifer and King (1933) for grasshopper eggs. The embryos were sectioned at 5 μ and stained with Giemsa, Wolbach's method.

Carnoy-Lebrun solution has the advantages of penetrating rapidly and at the same time removing the oil globules which become very hard if fixed in the yolk. The yolk is left rubbery by long fixation in Carnoy-

Lebrun although it is made very brittle by most fixatives. It usually becomes so hard during the process of dehydration and embedding that it will not section by the collodion-paraffin method or otherwise without long soaking. After Carnoy-Lebrun fixation, the hardening is not so severe and the soaking process is more effective than after most fixatives. Water penetrates the yolk at the rate of approximately one millimeter in 24 hours at 25° C., softening and slightly swelling it. Giemsa stain, well differentiated, gives fair contrast; chromatin blue-black, cytoplasm pale blue, yolk pink, and bacteroids blue-green. The Delafield hæmatoxylin-safranin method is unsatisfactory to demonstrate clearly the bacteroids in the embryo, as are all other stains tried.

Direct smears of fat bodies, gonads and embryos were used extensively for studying the morphology and checking for the presence of the bacteroids. Such smears were usually dried in air, fixed with heat and stained by Gram or Giemsa. Some were fixed in ether-alcohol, Bouin or Flemming while still wet, and stained as above. Other bacteriological stains were tried with less satisfactory results.

Morphology and Staining Reactions

The intracellular bacteroids of *Periplaneta americana* are slightly curved rods, .9 to 1 μ in diameter and 1.5 to 6.5 μ in length (average 3.5 μ), with round or slightly pointed ends. Spherical forms are of rare occurrence, most of the apparent cocci being ends of rods. In smears fixed in ether-alcohol, and occasionally in other fixatives, there is a distinct enlargement of one end of the rod, producing a club effect (Glaser, 1930*a*), but this is probably due to uneven shrinking and swelling in the fixative. No distinct size variation in the bacteroids has been found in different roaches of the same species.

The rods are either solid or have alternate dark and light bands (Fig. 2). The banded structure is most distinct in air-dried smears stained in Giemsa, and sections of fat-body or ovary fixed in Champy or Benda and stained with Haidenhain's iron hæmatoxylin, well extracted. Smears stained by most bacteriological methods show the banded effect indistinctly. Sections from material fixed in Carnoy-Lebrun, Bouin, Zenker, and other acetic acid fixatives show the banding in approximate inverse proportion to the amount of acetic acid in the fixative. In such material, many of the rods appear to be mere shells, while others have light bands (Fig. 2*b*).

The general size and appearance of the bacteroids is the same in all species of roaches studied. In *Blatta orientalis* and all *Parcoblatta* species studied, however, they are more slender, averaging about .9 by 3.5 μ, while in *Blattella germanica* they are shorter, thicker (1.1 by 3 μ)

and more nearly straight. These differences may be specific but such different roaches as *Periplaneta americana* and *Cryptocercus punctulatus* have bacteroids morphologically very similar.

The bacteroids obviously increase by transverse fission, as all stages of the process (Fig. 2) can readily be found in ovaries and embryos, although the division has not been observed directly. The constriction occurs regularly between two dark bands, dividing the original rod into two equal or unequal parts. The cocci and small buds mentioned by Glaser (1930*b*) have not been observed. Two, three or even four members may remain attached to make a chain as much as 15 μ long. The tendency to form chains is especially pronounced in *Parcoblatta*.

The bacteroids are non-motile, not encapsulated and not spore-forming. They are Gram-positive but not acid fast according to Glaser (1930*b*) and Neukomm (1927). With Sterling's Gram stain, however, they lose the violet completely after 80 to 90 seconds in alcohol, and with Kopeloff and Beerman's Gram stain they are usually negative, while *Bacillus subtilus* and *Staphylococcus aureus* mixed with the bacteroids retain the violet. They stain well with crystal violet, basic fuchsin, and mixtures containing either of these, but stain very poorly with methylene blue in any mixture. They stain readily with Giemsa, the periphery and bars becoming almost black in smears or deep blue-green in sections after differentiation in alcohol. They stain deeply with Haidenhain's iron hæmatoxylin, the intensity of the stain varying wth the fixative used. They do not give the Feulgin reaction to any definite degree. With Altman's acid fuchsin-methyl green the bacteroids stain green or blue-green, the chromatin green and the mitochondria red. The periphery of the bacteroids darkens slightly and unevenly after prolonged treatment in osmic acid.

In living material, the bacteroids are readily visible without stain. They do not take neutral red although the mycetocytes become pink after a short time. They stain distinctly with Janus green B in dilutions up to 1 to 100,000, but are not well differentiated with this stain at any dilution. Cowdry and Olitsky (1922) found no bacteria that stain in Janus green B in dilutions greater than 1 to 60,000, but mitochondria stain perfectly in dilutions as great as 1 to 500,000. They also found that mitochondria do not stain with Giemsa after any fixative used, nor with any stain used after alcohol or Bouin fixation.

With respect to morphology and reactions to fixatives and stains, the intracellular bacteroids and bacteria are very similar, to say the least. Neukomm (1927*b*) found that the reaction of the bacteroids of *Blattella germanica* to ultraviolet rays was likewise similar to that of bacteria. Glaser (1930*b*) has carried this analogy further, apparently with good reason, and has placed the bacteroids of the roaches taxonomically with the diphtheroids (genus *Corynebacterium*).

LIFE CYCLE

The following descriptions of the locations and behavior of the bacteroids throughout the life cycle of the roach agree in general with those given by Heymons (1895) and Buchner (1912). Many details are added, however, and some discrepancies in the works of former authors are clarified.

In roaches of both sexes, the spaces around the abdominal viscera are more or less filled with a lobulated fat body, well supplied with tracheæ. Each fat body lobe is made up ordinarily of two types of cells: a central row (mycetocytes) whose cytoplasm is packed with the bacteroids, and an outer layer of cells filled with fat globules and urate crystals, completely surrounding the mycetocytes (Fig. 3). Some lobes have two layers of cells around the mycetocytes: an inner layer filled with urate crystals which are usually dissolved during the process of fixation leaving the cytoplasm clear, and an outer layer of regular fat cells (Fig. 4; Cuénot, 1896, his Fig. 2). In thicker portions of the fat body there may be several rows of mycetocytes, interspersed with urate cells or fat cells and completely surrounded with fat cells. In *Periplaneta, Eurycotis,* and *Blatta,* many lobes are thin with only one row of mycetocytes. In *Blatella, Parcoblatta* and *Cryptocercus* more lobes are thicker, with two to twenty mycetocytes in a cross-section.

Mycetocytes, unaccompanied by fat cells, are present in the connective tissue covering both ovaries and testes, but no bacteroids have been found within the testes. In the ovaries of all species studied, however, there is a layer of bacteroids around the larger oöcytes, the relative thickness of which, in *Periplaneta americana,* is shown in Fig. 1.

Before attempting to discuss the relations of the bacteroids to the oöcytes, it seems best to describe some salient points of the structure of the ovary.

An ovary of *Periplaneta americana* is made up of eight tapering ovarioles each of which consists of three parts: an anterior "terminal filament" which anchors the ovary to the body wall, an elongated egg tube, and a short tubular pedicel connecting the posterior or basal end of the egg tube to the oviduct. The entire ovariole is enclosed in a tough, non-cellular membrane, the tunica propria, outside of which is a thin covering of connective tissue and mycetocytes.

The egg tube is divided into two parts: an anterior "germarium" or "tip" made up of oöcytes in the early stages of differentiation; and the " zone of growth " or " vitellarium " in which the oöcytes grow to maturity (Fig. 1). The most anterior cells in the zone of growth, designated in this paper as the " distal oöcytes," are distinctly larger and more rounded than those in the tip. As the distal oöcytes enlarge, the follicle cells scattered between them multiply and gradually form a complete epithelium around each oöcyte.

The oöcyte at the base of each ovariole reaches a maximum length of .7 mm. before the last moult, after which it enlarges rapidly to 1 mm. in length, then more slowly to about 2 mm. if copulation is not effected. Within an average of eight days after copulation, the basal oöcyte reaches its final size (1 by 3 mm.), becomes encased in a chitinous-like chorion and is oviposited. Meanwhile, the next three oöcytes anteriorly have increased to 1 mm., .6 mm. and .5 mm. in length respectively. Oviposition of the basal oöcyte seems to be the stimulus for more rapid enlargement of the next in line, so there is a succession of mature eggs at intervals of approximately eight days.

No bacteroids have been found in the germarium. There are usually a few in the angles between the distal oöcytes, and gradually increasing numbers more posteriorly, pressed closely against the oöcyte membrane (Fig. 5). The follicular epithelium forms outside the bacteroid layer and splits the layer between oöcytes thus enclosing a separate layer around each oöcyte.

The bacteroids continue to increase making a compact layer of unit thickness (Fig. 6). Then they pile up two or three thick, or more often they become arranged perpendicularly against the oöcyte membrane, giving the appearance in section, of an irregular pallisade (Fig. 7). The pallisade formation is usually complete in oöcytes of .09 by .11 mm. The layer becomes so compact that it buckles at both ends of the .11 by .4 mm. oöcytes, making ridges and knobs that project into the yolk (Fig. 8). During the course of the rapid growth of the egg from .6 mm. to 1 mm. in length, the bacteroid layer along the sides is stretched again to one or two bacteroids thick while the ridges at the ends enlarge and push farther into the yolk (Fig. 9). In the final more rapid growth period the lateral bacteroid layer is stretched more and is finally broken into small clumps which almost invariably lie in the angles between the ends of the follicle cells. The ridges of bacteroids at the poles of the 1 mm. oöcyte are principally broadened in this part of the growth period, forming at each pole an irregular disc-shaped mass approximately 15 μ thick at the center and 150 μ across.

If development of eggs is delayed by lack of copulation, dormancy of ovary during the non-reproductive stage, improper food, or by any other cause observed, the bacteroids do not become noticeably more

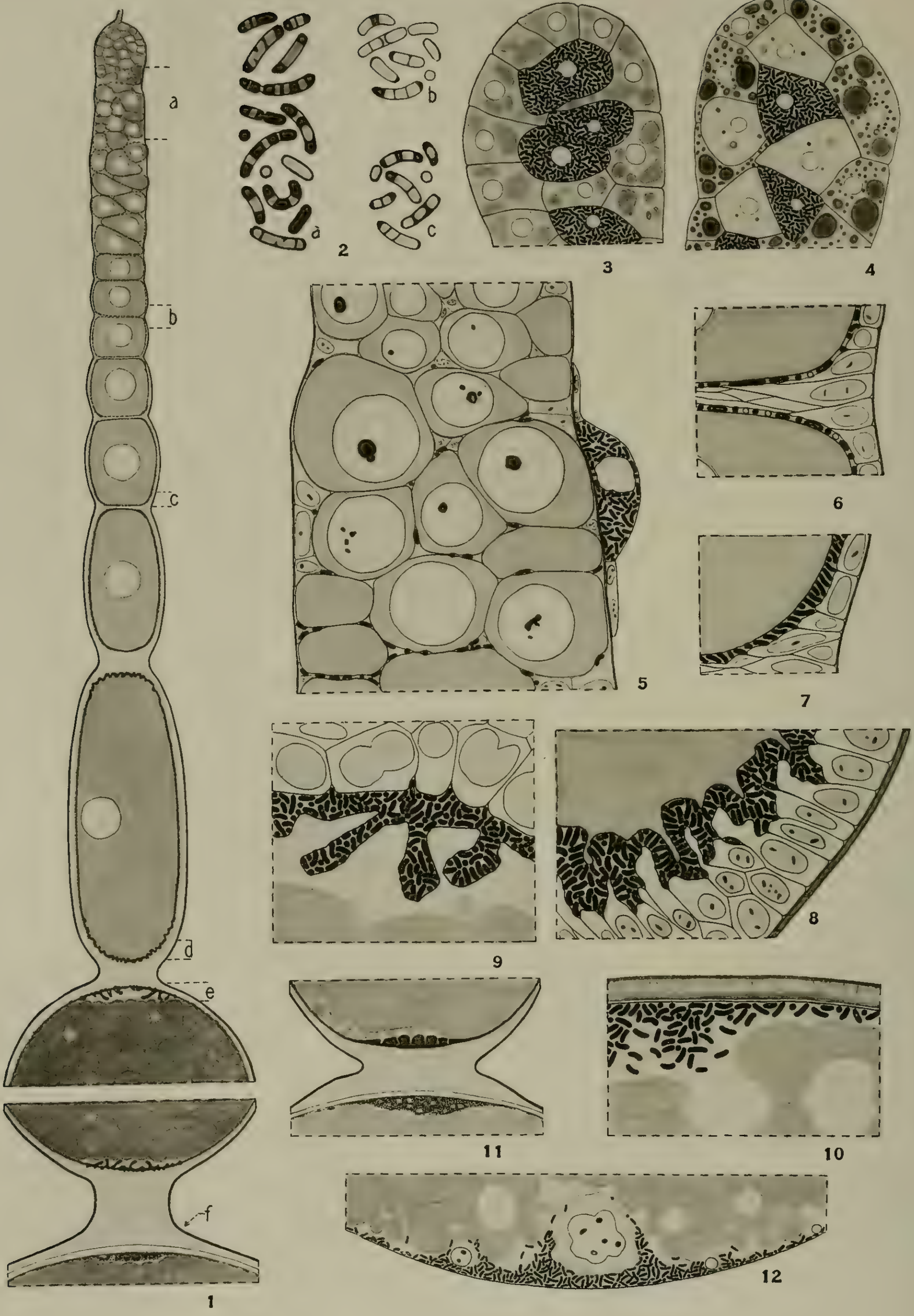
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PLATE I

abundant than in eggs of healthy, normally fertilized reproductive females.

Throughout the ovarian history described, the bacteroids are definitely between the oöcyte membrane and the follicle cells. They have not been found within the follicle cells in any species, as described by Buchner (1912), although single bacteroids are often found isolated between the follicle cells (Fig. 8). These probably have become separated from the bacteroid layer since they are always found between the inner ends of the follicle cells. In many preparations the follicle cell

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Explanation of Plates

All figures were made from camera lucida sketches at the stated magnifications and reduced one-half in reproduction. Unless otherwise specified, the figures are from *Periplaneta americana* material: embryos fixed in Carnoy-Lebrun and stained with Giemsa; and other material fixed in Champy and stained with Haidenhain's iron hæmatoxylin.

For the sake of clearness and contrast, cellular details are omitted and the bacteroids depicted in solid black regardless of their appearance in the section. In the drawings of embryos, the areas occupied by bacteroids are heavily stippled.

Plate I

Explanation of figures

Fig. 1. A mature ovariole, schematized and parts of the large eggs omitted, showing the relative thickness of the bacteriod layer around the oöcytes. The lettered rulings at the side indicate positions from which Figs. 5 to 10 were taken. × 150.

Fig. 2. Typical bacteroids from around oöcytes, stained with Haidenhain's iron hæmatoxylin after fixation in (*A*) Champy, (*B*) Carnoy-Lebrun, and (*C*) Flemming. × 3,000.

Fig. 3. Typical fat body lobe from *Blatta orientalis,* Carnoy-Lebrun fixation. × 650.

Fig. 4. Fat body lobe with urate cells around the mycetocytes. × 650.

Fig. 5. Higher magnification of segment *a,* Fig. 1. Bacteroids around the distal oöcytes and in a mycetocyte at the side of the ovariole, outside the tunica propria. × 1,100.

Fig. 6. Higher magnification of segment *b,* Fig. 1. Bacteroids as a single layer between oöcytes and follicle cells. × 1,500.

Fig. 7. Higher magnification of segment *c,* Fig. 1. Beginning of pallisade formation. × 1,500.

Fig. 8. Higher magnification of segment *d,* Fig. 1. Bacteroid layer buckling at the end of .5 mm. oöcyte. × 1,500.

Fig. 9. Higher magnification of segment *e,* Fig. 1. Ridges of bacteroids at the end of a 1 mm. oöcyte, yet limited between oöcyte membrane and follicle cells. × 1,500.

Fig. 10. Higher magnification of segment *f,* Fig. 1. Bacteroids dispersing through the polar cytoplasm of a mature egg. × 1,500.

Fig. 11. Posterior end of 1 mm. oöcyte and anterior end of mature egg of *Blatta orientalis.* × 150.

Fig. 12. Bacteroid mass, with one giant nucleus and three smaller ones, at the posterior end of an egg after 60 hours incubation. × 650.

wall is very distinct both in well-fixed tissues and in shrunken material. The oöcyte membrane is very thin and is visible only as a definite limit to the cytoplasm.

Shortly before the egg is oviposited and soon after the first appearance of the chorion, the oöcyte membrane disappears and a new membrane, interpreted as the vitelline membrane, is formed between the bacteroids and the chorion (Fig. 10). The bacteroids, formerly limited to definite ridges, now disperse throughout the cytoplasm between the vitelline membrane and the yolk so that by 36 hours after oviposition there is a homogeneous disc-shaped mass of bacteroids at each pole, and numerous isolated clumps around the periphery of the egg. No bacteroids have ever been seen within yolk granules, as reported by Buchner (1912, his Fig. 6).

Buchner (1912) explained the polar concentrations by assuming an active migration of the bacteroids toward the poles. Such an assumption is not necessary if the differential rate of surface increase is considered. For example, an oöcyte 90 μ in diameter and 100 μ in length is surrounded by a uniform layer of bacteroids, while one 160 by 600 μ has very noticeable concentrations at the poles. Considering the oöcytes as cylinders, the ratio of end area to side area of the smaller is 6360:28,300 and of the larger is 20,100:301,600. This is an increase in end area of 201 per cent and in side area of 966 per cent; or, the side has expanded 4.8 times as much as the end. The original end of the oöcyte, however, enlarges at most to 140 μ, the remainder of the increase in diameter being accounted for by curving of the side, thus adding to the calculated growth of side surface. Consideration of the changing proportions during the final growth period is an even more convincing argument for this point. In enlarging from .35 by 1 mm. to 1 by 3 mm., the area of the sides increases approximately ten times while the original end of the egg expands less than 50 per cent. Without calculating the differential increases accurately, it is obvious that these differences are sufficient to account for the polar concentrations and the later thinning of the lateral layers, if the bacteroids multiply at the same rate all around the oöcyte.

In all species of roaches studied, the ovarian history of the bacteroids is remarkably uniform, the only differences being in the number of the bacteroids present and in the structure of the ovary itself.

Periplaneta australasiæ is like *Periplaneta americana* in all respects noted. *Eurycotis floridana* differs from these only in having smaller polar masses of bacteroids.

Blatta orientalis shows the same bacteroid relations around the smaller oöcytes, but the layer becomes much thicker, buckling distinctly in .3 mm. eggs. The resulting ridges increase to such an extent that at each pole of a .5 mm. egg there is a nearly solid hemispherical mass which enlarges to a maximum of 60 by 75 μ in a .7 mm. egg, then spreads out in the final growth period into a vacuolated disc about 40 μ thick and 160 μ in diameter (Fig. 11). The vacuolated appearance is due to a partial restitution of the original ridges of the bacteroids. *Cryptocercus punctulatus,* although it has the same type of ovary as *Periplaneta americana,* ordinarily does not have bacteroids around the first four to ten distal oöcytes. The bacteroid layer becomes 4 μ to 5 μ thick, with no tendency to form a pallisade arrangement. The layer buckles uniformly around the .6 mm. egg, with only slight polar concentrations until the side layers are thinned in the final rapid growth period. No mature eggs of this species were studied. Consistently in the *Cryptocercus punctulatus* specimens examined, about one-tenth of the young oöcytes were infected and apparently destroyed by the bacteroids. They penetrated the oöcyte membrane, usually in very early stages, and increased within the yolk until what had been an oöcyte was a mass of bacteroids. A similar condition is occasionally found in *Blatta orientalis* and rarely in *Periplaneta americana.* In these latter, however, the bacteroids never increase as much as in *Cryptocercus.* In *Blattella germanica* and the species of *Parcoblatta* studied, the ovarioles are very slender near the germarium, each containing only one oöcyte in cross-section in contrast to the two, three or four in a similar section of a *Periplaneta* ovariole. Accompanied with this difference is the absence of any bacteroids around the first two to four distal oöcytes. The most anterior one to have bacteroids always has the greater number at the posterior end. There are always two or more oöcytes surrounded by bacteroids anterior to a completed follicle, which here, as in *Periplaneta,* splits the layer between oöcytes. The bacteroids gradually become more numerous, making a double layer or an incomplete pallisade around the oöcyte. There may be a slight buckling at the corners and at most a triple layer at the poles. In the final enlargement of the egg, the bacteroids become scattered around the sides while the end concentrations remain as thin layers.

In all species of roaches studied, bacteroids invariably occupy the space between the follicle cells and the oöcyte until the vitelline membrane begins to form, at which time they enter the egg cytoplasm.

After oviposition, while the early cleavage nuclei are dividing within the yolk, the bacteroids retain their position against the vitelline membrane. Many of the first nuclei to reach the periphery (two days incubation), enter portions of cytoplasm containing bacteroids, and within a few hours sink again into the yolk accompanied by the bacteroids.

These nuclei, with their cytoplasm and bacteroids, I shall call "primary mycetocytes." Others, indistinguishable from the above, come to the periphery and return into the yolk without bacteroids. These are the yolk nuclei or vitellophags. The remainder of the nuclei stay at the surface and form the blastoderm, which is continuous by the third day, except at the regions of the polar masses of bacteroids.

The course of events at the poles is not so simple as that for the rest of the egg surface. Each polar mass (Fig. 12) receives five to ten nuclei toward the end of the second day of incubation. The number increases to about forty by the fourth day. Most of them resemble the primary mycetocyte nuclei but a few (one to four) of the original ones become very large and have a considerable volume of clear cytoplasm. Apparently these giant nuclei do not divide and their later history is unknown except that they become separated from the bacteroids early in the migration of the polar masses. After this, they are not readily distinguishable from the ordinary vitellophags.

On the third day, the anterior bacteroid mass breaks into several smaller clumps which within two days sink into the yolk, reunite and proceed toward the center of the egg. The posterior bacteroid mass at

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PLATE II

Explanation of Figures

FIG. 13. Posterior bacteroid mass passing ventrally and anteriorly between the yolk and the embryonic rudiment. One giant nucleus has been left behind. Posterior ventral segment of a median sagittal section of an egg after 3½ days incubation. × 150.

FIG. 14. Bacteroid masses in the yolk. The outlines of the yolk granules are shown only around the bacteroid clusters. This and the following eggs have yolk granule and oil globule arrangement similar to that of Fig. 13. Median sagittal section of a six-day embryo. × 55.

FIG. 15. Bacteroid masses uniting near the center of the yolk to form the primary mycetome. Median sagittal section of a nine-day embryo. × 55.

FIG. 16. Eleven-day embryo with the primary mycetome still intact. × 55.

FIG. 17. Longitudinal section of the last thoracic segment and the anterior part of the abdomen of a 12-day embryo, nearly full length of the ovary. Approximately at the location of guide line *b* on Fig. 18. Many cells of the fat body contain bacteroids. × 110.

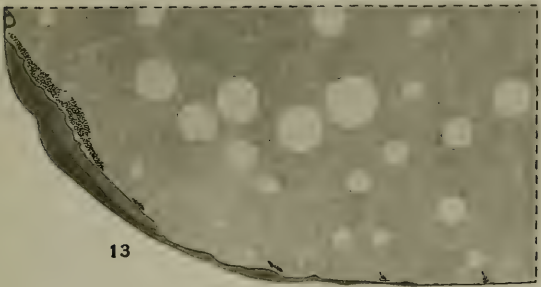
FIG. 18. Cross-section through the third abdominal segment of 12-day embryo (from the level of *a* on Fig. 17). × 110.

FIG. 19. *Parcoblatta pennsylvanica* embryo at approximately the same stage of development as that in Fig. 14. Bacteroids moving in small streamers between the yolk granules. × 70.

FIG. 20. *P. pennsylvanica* embryo intermediate in development between those of Figs. 15 and 16. Bacteroids in the primary mycetome. × 70.

FIG. 21. Portion of an ovariole of a four-month-old *Periplaneta americana*. Bacteroids are located in the mycetocyte outside the tunica propria and as a small clump between two oöcytes around which they are dispersing. × 1,000.

FIG. 22. Median section through the mycetome of a ten-day embryo. × 300.



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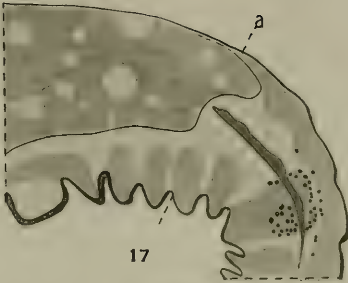

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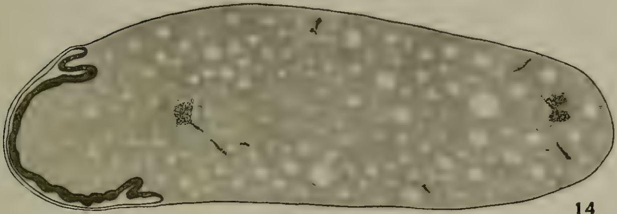

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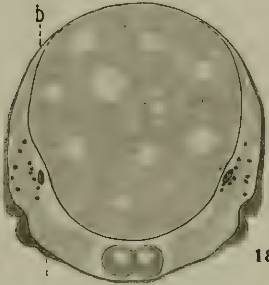

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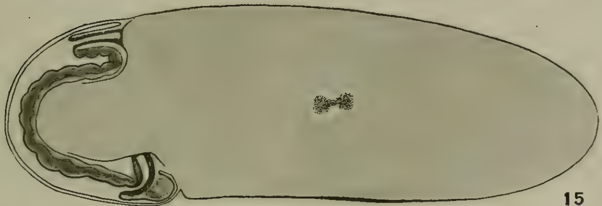

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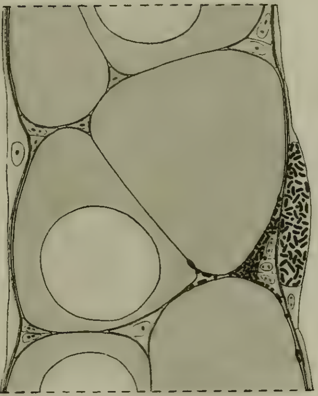

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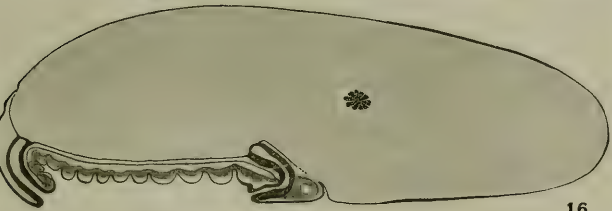

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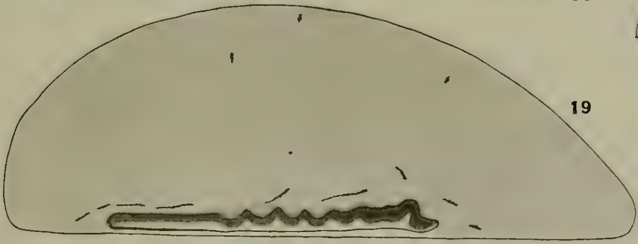

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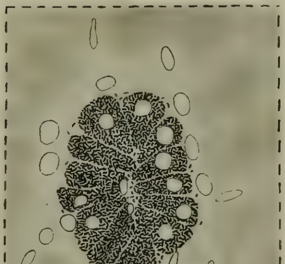

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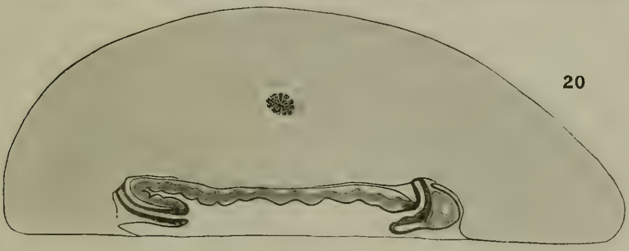

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PLATE II

this time is nearly spherical and lies at the posterior end of the newly differentiated embryonic rudiment. Near the end of the third day this mass begins to migrate anteriorly and ventrally, passing between the germ band and the yolk to about the middle of the embryo (Fig. 13), then it swings directly into the yolk (fifth day), either as a solid mass or as several clumps which reunite after proceeding a short distance. Both polar masses now move slowly toward the center of the yolk (Fig. 14) being joined on the way by the small lateral masses. All bacteroid masses join just anterior to the center of the egg about the end of the eighth day (Fig. 15) to form a compact mass approximately .1 mm. across, which mass Buchner (1912) called the " primary mycetome." The bacteroids within this mycetome are arranged in pyramidal segments, fitted together to form a sphere (Fig. 22). Each segment has one or two nuclei. Around the bacteroid sphere there is a single to double layer of vitellophag nuclei. No cell walls have been distinguished in this mass.

Heymons (1895) believed that the nuclei associated with the bacteroids increase by amitotic division. Although mitotic figures are rarely found and the nuclei are often lobulated as if pinching in two, I am not convinced that there are not enough mitotic divisions to account for the very slow increase in the number of these nuclei.

There is no question that the bacteroids do exhibit definite migrations. The posterior bacteroid mass in some eggs moves as much as 2.5 mm. in five days. This mass is made up of a loose clump of bacteroids and mycetocyte nuclei, surrounded by a layer of vitellophags. In some eggs the anterior surface of the mass is very irregular as if it were pushing forward between the yolk granules. In others, the yolk in the path over which the bacteroids have passed is more homogeneous than the surrounding yolk, suggesting that it had been dissolved. The small lateral masses unquestionably flow inward between the yolk granules in company with one or two ameboid nuclei. The bacteroids in all cases appear to be carried along passively with the mycetocytes and vitellophags.

So far as can be determined, all bacteroids in the egg are enclosed in the primary mycetome for about three days, after which some apparently leave the mycetome and migrate to the embryo.

At eleven days incubation the embryo has straightened out along the ventral surface of the egg and is approximately one-half the length of the egg (Fig. 16). Segmentation is complete on the ventral side, but the dorsal wall is formed only on the most posterior three or four abdominal segments. The lateral walls are formed dorsally to a line running diagonally around the egg from the head to the most anterior point of the completed dorsal wall. The yolk is rapidly enclosed from this time on by dorsal and anterior growth of the lateral walls and elongation of the whole embryo so that by twelve days the dorsal wall is formed to the third abdominal segment (Fig. 17), and by fifteen days the embryo is as long as the egg and the yolk is entirely enclosed. At eleven days the fat bodies are rudimentary and strictly segmental, while the gonads are elongated masses of cells. By twelve days the gonads are definite cords, and by thirteen days the ovariole rudiments are recognizable as segmental enlargements of the ovary. The gut epithelium is continuous along the ventral side of the yolk between the stomodeum and the proctodeum and extends dorsally around the yolk as a discontinuous cell layer. The body cavity ends blindly at its anterior margin beyond the limits of the gut epithelium (Figs. 17, 18).

In the period between eleven and twelve days of incubation, the bacteroids within the mycetome lose their definite arrangement. The mycetome elongates and breaks and bacteroids appear between the yolk granules posterior to the mycetome. They also appear against the gut epithelium, in the body cavity, and in cells of the lateral lobes of the fat body bordering the body cavity. Immediately after the mycetome breaks, its posterior wall appears to have moved into the yolk. After eleven days of incubation, the number of bacteroids against the gut epithelium increases, reaching a maximum at about the eighteenth day. After that time they rapidly disappear from the gut, apparently by degeneration. The nuclei of the mycetome become indistinguishable from the vitellophags, and apparently most if not all of them fuse with the gut epithelium late in embryonic development to become the definitive entoderm, much as described by Stuart (1935) for a grasshopper.

The foregoing description of the primary mycetome, behavior of the bacteroids, and embryonic development in general, are matters of direct observations on fixed and stained material. Since it is impossible to see the bacteroids within the dense, living egg, their behavior must necessarily be interpreted from static material.

Heymons (1895) stated that the bacteroids move centrifugally from the primary mycetome through the wall of the gut and into certain cells of the fat bodies, after the gut completely encloses the yolk. Considering the stage of embryonic development and the places in which the bacteroids are found at the time, the following method of infection seems most probable. The vitellophags liquify the yolk around the primary mycetome which then loses its compactness. This liquid digestion product suddenly flows toward the embryo, separating the posterior cells of the mycetome. The nuclei are detained by the dense yolk granules, but some of the bacteroids are carried along passively in the flow until they reach the embryo. If these bacteroids come in contact with the formed gut epithelium, they are retained while the liquid that carried them diffuses through. If they come in contact with the incomplete margin of the gut, or pass immediately anterior to the gut, they enter the body cavity directly. Within the cavity they are carried more posteriorly and ventrally and brought into contact with the fat bodies.

It seems possible, however, that in the early migration of the bacteroids a few may have remained with the embryonic rudiment while the rest proceeded to the primary mycetome.

Most, if not all, of the cells of the lateral lobes of the abdominal fat bodies bordering the body cavity take up a few bacteroids in the cytoplasm between the cell nucleus and the body cavity, thereby becoming mycetocytes. No differences between the cells of different parts of the fat bodies are discernible before infection, nor has any mechanical principle been found that would explain selective infection, yet the fact remains that neither the dorsal nor the ventral parts of the fat bodies become infected. As development proceeds, uninfected fat cells inclose the mycetocytes, or possibly as Buchner (1912) thinks, the infected cells sink into the fat body. The segmental fat bodies enlarge and fuse, having completely enclosed the gonads in the meantime. The bacteroids within each mycetocyte multiply rather slowly, causing an increase in mycetocyte size from approximately 12 μ at the time of infection to 25 μ at the time of hatching, and 30 μ at the first moult. Few mycetocytes get larger than 35 μ in diameter. The mycetocyte nuclei remain distinctly eccentric until two or three weeks after the roach hatches. No indication of mycetocyte division has been found, and no increase in numbers of mycetocytes in a roach is discernible after the first infection. The final form of the fat body is attained by the time of the second moult by two processes; dispersal of the mycetocytes among the ever enlarging fat cells before the roach hatches; and later separation of the mass into lobes of varying sizes, most of which have cores of mycetocytes.

Infection of the ovary has been considered to be a more complicated process than has that of the fat body. Buchner (1912) considers it probable that the bacteroids pass directly from the mycetocytes (Fig. 5 and Buchner, 1912, his Fig. 1), through the young follicle cells to the oöcytes. Fraenkel (1921) reported that she has seen steps in this process (her Figs. 1, 2). They do not specify, but intimate that this process is repeated for the infection of each oöcyte. It seems improbable, however, that a non-motile element could pass through the wall of the mycetocyte, across a potential cavity, through the very tough tunica propria and finally through the follicle cell.

The relationships between bacteroids and oöcytes described above (p. 439) exist in immature ovaries as young as four months (second moult). The ovaries of a young first moult roach are all like the tips of older ovarioles. Each ovariole is closely surrounded by mycetocytes outside the tunica propria. A cursory examination of the ovary at this stage reveals no bacteroids within the tunica. Before the second moult, a few oöcytes in the posterior part of each ovariole have enlarged to two or three times their original diameter. Against some of these, there are clumps of bacteroids apparently dispersing around the oöcytes (Fig. 21). These groups of bacteroids are often very close to mycetocytes from which they sometimes appear to be flowing, but no break in the tunica propria has been found.

Careful study of ovaries of roaches younger than three months almost invariably reveals the presence of one or more bacteroids in each ovariole, usually among the more posterior oöcytes. The bacteroids in this stage are easily overlooked because they stain much like the chromatin of the oöcyte nuclei. Either they occur in greater numbers or they are more easily recognized between the enlarging oöcytes of the three-month ovaries than between the small oöcytes of the one-month ovary.

In consideration of the observations just given, the following is the most probable method of infection. At the time of the fat body infection, a few bacteroids find their way into the mass of ovarian cells and remain there, increasing very slowly for a time. Within twenty-four hours the ovary becomes a compact cord of germ cells, between which are scattered the bacteroids. The ovarioles are formed as outgrowths from the original cord, enlarging at first entirely through multiplication of the oögonia. As this multiplication is greatest at the distal end, the bacteroids there become more scattered. The oögonia at the basal end of the ovariole soon cease division. Their chromatin passes through pachyphase before the roach hatches, and becomes diffuse soon after the first moult. With the advent of the diffuse chromatin the oöcyte begins more rapid growth and the bacteroids begin dividing and gradually spread over the surface of the oöcytes (Fig. 21). This dispersal is undoubtedly accelerated by the shifting of the oöcytes in the ovarioles and by pressure on the ovariole from general body movements. With the completion of the follicle around the most posterior oöcytes, the spread of bacteroids is limited to a forward migration which keeps pace with the development of new oöcytes to the infectable stage.

In review, the following observed facts favor this hypothesis of ovarian infection: (1) the ovary is a rough mass of cells without definite borders at the time of fat body infection; (2) there are a few bacteroids in the very young ovary; (3) the first obvious infections are small clumps of bacteroids which appear to be spreading around the oöcytes. These clumps may be in the center of the ovariole, far removed from a mycetocyte; (4) passage of bacteroids from mycetocytes to ovarioles has not been convincingly demonstrated; (5) there are always several oöcytes with bacteroids at their surfaces anterior to a completed follicle; and (6) in ovarioles like those of *Periplaneta,* there are usually bacteroids around all oöcytes posterior to the tip, and so arranged as to give the impression that they had moved anteriorly in the angles between the oöcytes, while in ovarioles like those of *Parcoblatta* where each oöcyte fills the entire cross-section of the egg tube, the bacteroids are arranged evenly around the most anterior infected oöcyte.

The principal points against this hypothesis are: (1) the migrations of the bacteroids have not been seen in living roaches; (2) no bacteroids have been seen in the ovary for two weeks after the supposed time of infection (possibly due to lack of differential staining); and (3) the hypothesized retardation of bacteroid growth in the early ovary has not been established. This last objection, however, is apparently the same factor that limits the bacteroids to oöcytes that have passed a certain stage of development.

No differences have been found in the bacteroid behavior throughout the embryonic development of *Periplaneta americana, P. australasiæ* and *Eurycotis floridana.* However, *Blatta orientalis, Parcoblatta pennsylvanica, P. uhlerania,* and *P. latta* differ from *Periplaneta* in the effect of a median ventral rather than a posterior embryonic rudiment. In *Parcoblatta* the polar masses migrate directly into the yolk. In this migration, the bacteroids may be in small separated clumps, each with two to four nuclei, and pass close to the embryo (Fig. 19), or they may be in a large loose mass and pass deeper through the yolk. The lateral clumps join the general mass while en route but remain distinct until they reach the center of the egg. Here all masses unite to form a primary mycetome (Fig. 20) very similar to that of *Periplaneta americana.* In *Blatta orientalis* the polar masses are much larger, but the behavior is similar to that in *Parcoblatta.* Bacteroid behavior after formation of the primary mycetome is the same in all species studied, so far as could be determined. I have no embryological material of *Cryptocercus punctulatus* and so little of *Blattella germanica* that it warrants no comparisons nor conclusions as to bacteroid behavior. Buchner (1912) described a primary mycetome in the last-named species.

I wish to express my gratitude to Dr. Fernandus Payne and to the other members of the Faculty of the Zoölogy Department of Indiana University for their criticism and suggestions during the progress of this work.

SUMMARY

1. The staining reactions, morphology and occurrence both intra- and intercellularly definitely preclude the intracellular bacteroids of the roaches from the category of mitochondria and link them closely to both the diphtheroids and the Rickettsia.

2. The number of bacteroids between the oöcyte membrane and the follicle cells increases until there is a uniform layer two or three bacteroids thick. By differential increase of the oöcyte surface this layer becomes thinner along the sides.

3. Before the egg is oviposited, the original oöcyte membrane breaks down and permits the bacteroids to enter the cytoplasm.

4. As the embryo develops, the bacteroids, accompanied by nuclei similar to the vitellophag nuclei, move in masses to the center of the yolk.

5. Later a few bacteroids from this central mass move posteriorly between yolk granules through the incomplete margins of the gut epithelium into the body cavity. From there most of them are taken up by the cells of the lateral lobes of the fat bodies while a few are caught between the cells of the forming ovaries.

6. The bacteroids within the ovariole seem to lie dormant for several weeks, then they multiply rapidly and spread over the surfaces of the enlarging oöcytes.

7. Newly enlarged oöcytes from the germarium are infected by bacteroids moving anteriorly from older oöcytes.

8. Bacteroid behavior varies between roach species with differences in number of bacteroids, in structure of the ovary, and in position of development of the embryonic rudiment.

9. All bacteroid migrations appear to be passive.

LITERATURE CITED

Blochmann, F., 1888. Ueber das regalmässige Vorkommen von bakterienähnlichen Gebilden in den Geweben und Eiern verschiedener Insecten. *Zeitschr. f. Biol.,* **24**: 1.

Buchner, P., 1912. Studien an intracellularen Symbionten. I. Die intracellularen Symbionten der Hemipteren. *Arch. f. Protist.,* **26**: 1.

Buchner, P., 1921. Tier und Pflanze in intracellularer Symbiose. G. Bornträger, Berlin.

Buchner, P., 1923. Studien an intracellularen Symbionten. IV. Die Bakteriensymbiose der Bettwanze. *Arch. f. Protist.,* **46**: 225.

Buchner, P., 1925. Intracellulare Symbiose der Tiere mit pflanzlichen Mikroorganismen. *Handb. d. biol. Arbeitsmeth.*, Abt. XII, Tiel **1**: 325.

Buchner, P., 1930. Tier und Pflanze in Symbiose. 2 Aufl. G. Bornträger, Berlin.

Cowdry, E. V., and P. K. Olitsky, 1922. Differences between mitochondria and bacteria. *Jour. Exper. Med.*, **36**: 521.

Cuénot, L., 1896. Études physiologiques sur les Orthoptères. *Arch. Biol.*, **14**: 293.

Florence, L., 1924. An intracellular symbiont of the hog louse. *Am. Jour. Trop. Med.*, **4**: 397.

Fraenkel, H., 1921. Die Symbionten der Blattiden im Fettgewebe und Ei insbesondere von Periplaneta orientalis. *Zeitschr. f. wiss. Zool.*, **119**: 53.

Glaser, R. W., 1930*a*. The intracellular "symbionts" and the "Rickettsia." *Arch. Path.*, **9**: 71–96 and 557–576.

Glaser, R. W., 1930*b*. On the isolation, cultivation and classification of the so-called intracellular "symbiont" or "Rickettsia" of Periplaneta americana. *Jour. Exper. Med.*, **51**: 59.

Henneguy, L., 1904. Les insectes. Mason et Cie, Paris.

Hertig, M., 1921. Attempts to cultivate the bacteroids of the Blattidæ. *Biol. Bull.*, **41**: 181.

Heymons, R., 1892. Die Entwicklung der weiblichen Geschlechtsorgane von Phyllodromia (Blatta) germanica L. *Zeitschr. f. wiss. Zoöl.*, **53**: 434.

Heymons, R., 1895. Die Embryonalentwicklung von Dermapteren und Orthopteren. Gustav Fischer, Jena.

Koch, A., 1931. Die Symbiose von Oryzaephilus surinamensis L. (Cucujidæ, Coleoptera). *Zeitschr. f. Morph. u. Ökol. Tiere*, **23**: 389.

Lilienstern, M., 1932. Beiträge zur Bakteriensymbiose der Ameisen. *Zeitschr. f. Morph. u. Ökol. Tiere*, **26**: 110.

Mansour, K., 1934. On the intracellular micro-organisms of some bostrychid beetles. *Quart. Jour. Micr. Sci.*, **77**: 243.

Mansour, K., 1934. On the so-called symbiotic relationship between coleopterous insects and intracellular micro-organisms. *Quart. Jour. Micr. Sci.*, **77**: 255.

Mercier, L., 1907. Recherches sur les bactéroïdes des Blattides. *Arch. f. Protist.*, **9**: 346.

Meyer, K. F., 1925. The "bacterial symbiosis" in the concretion deposits of certain operculate land mollusks of the families Cyclostomatidæ and Annulariïdæ. *Jour. Infect. Dis.*, **36**: 1.

Neukomm, A., 1927*a*. Sur la structure des bactéroïdes des Blattes (Blattela germanica). *Compt. rend. Soc. Biol.*, **96**: 306.

Neukomm, A., 1927*b*. Action des rayons ultra-violets sur les bactéroïdes des Blattes (Blattela germanica). *Compt. rend. Soc. Biol.*, **96**: 1155.

Schwartz, W., 1924. Untersuchungen über die Pilzsymbiose der Schildläuse. *Biol. Centralbl.*, **44**: 487.

Slifer, E. H., 1934. Insect development. VI. The behavior of grasshopper embryos in anisotonic, balanced salt solutions. *Jour. Exper. Zoöl.*, **67**: 137.

Slifer, E. H., and R. L. King, 1933. Grosshopper eggs and the paraffin method. *Science*, **78**: 366.

Stuart, R. R., 1935. The development of the mid-intestine of Melanoplus differentialis (Acrididæ: Orthoptera). *Jour. Morph.*, **58**: 419.

Wolbach, S. B., 1919. Studies on Rocky Mountain spotted fever. *Jour. Med. Res.*, **41**: 1.

AUTOGENOUS TRANSPLANTATIONS OF PIGMENTED AND UNPIGMENTED EAR SKIN IN GUINEA PIGS[1]

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The experiments to be described consist in a continuation of a series of investigations begun by Loeb and by Carnot and Deflandre, and studied further by Sale and Seelig and others, concerning the fate of autogenous transplants of pigmented and unpigmented skin in the ear of the guinea pig. Carnot and Deflandre (1896) and Loeb (1897) independently carried out experiments in which they transplanted pigmented and unpigmented epidermis from the ears of guinea pigs. In certain respects the results obtained by these investigators were similar, but in other respects differences developed. When unpigmented ear skin is autotransplanted to a defect in pigmented skin, the graft is either lost by scaling, or is invaded and replaced by pigmented epithelium from the surrounding epidermis. An autogenous graft of pigmented skin to a defect in a white area, however, persists; and in the course of time the pigmented cells grow out and invade the surrounding white epidermis, thus increasing the size of the colored graft. Although it is not especially stated, Loeb's transplants were autogenous, whereas Carnot and Deflandre state that they successfully carried out homoiogenous and even heterogenous grafts. The latter investigators furthermore report that while grafts from one species to another as a rule failed to grow, grafts from guinea pig to rabbit, and from rabbit to guinea pig, appeared to persist for a while, although with certain difficulties. These authors could not detect any differences in behavior between autogenous and homoiogenous transplants.

The investigations of Loeb were carried further by Sale (1913) and Seelig (1913), who compared autogenous and homoiogenous grafts of pigmented and unpigmented skin in guinea pigs. Sale showed that auto- and homoiogenous transplants of pigmented epithelium to white skin behave differently. The large majority of autogenous grafts survive and outgrowth of pigmented epidermis into the adjoining white

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epithelium is the rule. Only a few of the homoiogenous pigmented grafts remained alive, but in these cases invasion of the adjacent white epidermis did not occur. Instead, the pigment-producing function of the transplanted cells was injured and the graft gradually lost its pigment. As a rule an accumulation of lymphocytes underneath or in the transplanted epidermis preceded a partial separation of the transplant and its destruction in the majority of cases. Seelig has shown that unpigmented grafts when auto- or homoiotransplanted to defects in pigmented skin, are invaded and replaced by the adjacent pigmented epidermis.

Rand and Pierce (1932), studying autogenous and homoiogenous transplants of white ventral skin to pigmented areas in the tadpole, concluded from gross and microscopic observations that pigmentation of these grafts occurred through invasion and replacement of the graft by the surrounding epidermis. Barriers of hen's eggshell membrane could temporarily protect the graft from invasion and destruction. In autogenous grafts this invasion did not occur so regularly as in homoiotransplants. Herrick (1932) confirmed this observation so far as homoiogenous grafts in tadpoles were concerned, but concluded that autogenous grafts of unpigmented ventral skin retain their specificity and are not invaded for more than one hundred days.

Seevers and Spencer (1932) were unable to confirm the results of Loeb in regard to outgrowth of autogenous pigmented skin grafts into the surrounding white skin and the invasion of unpigmented grafts by surrounding pigmented epithelium in guinea pigs. Rectangular grafts having areas of about two square centimeters were made and the animals were observed for as long as nine months. During this time the transplanted black epidermis retained its pigment but it did not grow out into the neighboring white skin in any case. A few white grafts transplanted to defects in pigmented areas did become slightly darker along their borders after four to six months, but many such grafts remained unpigmented throughout the period of observation. In no case was the color of the hair changed, either in the graft or surrounding tissue. Except in the case of transplants to back and belly in agouti guinea pigs, there is no definite statement as to the part of the animal's body where these grafts were made; in the case of the experiments of Loeb and the other workers the ear was used without exception.

The technique used in our experiments was similar to that used by Sale and by Seelig. Guinea pigs were selected which had either one pigmented and one white ear, or sufficiently large pigmented and white areas on either one or both ears. With aseptic precautions a circular area of each type of skin approximately 4 mm. in diameter was removed with a sharp razor. As little as possible of the connective tissue underlying the epidermis was included in the excised tissue. Bleeding, usually slight, was controlled by pressure. The skin pieces removed were placed in 0.9 per cent sodium chloride solution and cut to the desired size, usually about 2 mm. square. After oozing had ceased the grafts were placed on the denuded areas according to the experiments described, blotted, and alcohol-soaked cotton dressings were firmly applied, held in place with collodion. The dressings were usually loosened at the end of ten days, and subsequently observations and measurements were made at intervals of a few days or weeks for several months. Thirty-two animals in all were used and eighty-three individual grafts were made. Approximately one-half of the grafts did not survive, probably owing to errors in technique in our experimental procedure, or more often perhaps owing to scratching of the wound on the part of the animals.

The following data give a brief summary of the experiments performed and the survival of the grafts. It is interesting to note that practically the same proportion of pigmented and unpigmented grafts survived.

	Total	Survived
White grafts to pigmented skin	34	15
Single pigmented grafts to white skin	24	10
Double pigmented grafts to white skin	11	5
Triple pigmented grafts to white skin	1	1

In making the double and triple pigmented grafts a small area of intact white skin was usually left between the defects. Multiple transplants of white to pigmented skin were also made, but as they did not behave differently from single transplants, they are included in the discussion of the latter. The behavior of some of the representative grafts is described below.

Autogenous Transplants of Unpigmented Skin to Defects in Pigmented Skin

Our observations in fifteen cases where we were certain that the grafts were present were in accord with those of Loeb, Carnot and Deflandre, and Seelig. Complete invasion of the graft occurred in all cases. During invasion by surrounding pigmented epithelium there was, we also noticed, some scaling of the superficial layers of the graft. This appeared to persist as long as any white epidermis was yet visible, but its exact degree could not be determined grossly. In Seelig's experiments the time required for complete invasion varied from 13 to 90 days. We observed complete invasion in one case at 24 days, and in the others the time required varied up to about ninety days or more. In one instance 200 days were required, and here the grafting was associated with considerable thickening of the underlying tissues, and this reaction, or perhaps the condition which gave rise to it, may have injured the host epidermis and thus have been responsible for the slow growth.

After complete invasion the site of the graft was usually somewhat thickened and appeared more deeply pigmented than the rest of the ear. Since similar observations have been made in connection with normal regeneration, this perhaps indicates that the presence of a graft of unpigmented epidermis does not greatly alter the normal regenerative processes of pigmented epidermis except in so far as rate of repair is concerned.

No correlation between the rate of invasion and the color or degree of pigmentation of the skin surrounding the transplant could be noted, grafts being made in defects of black, gray and brown skin.

Single Autogenous Transplants of Pigmented Skin to Defects in Unpigmented Skin

The results of these experiments likewise confirm the earlier observations of Loeb, Carnot and Deflandre, as well as those of Sale. Outgrowth of pigmented epidermis from the graft occurred in each of the ten cases studied. Some of these grafts were observed for 280 days. An average of about twenty days is required before a recognizable increase in the size of the pigmented area occurs. Occasionally the graft shriveled somewhat in this period, and in two cases seemed to have been lost. However, in these two cases a small point of pigment could be recognized at the center of the defect after 10 days and subsequently grew at a rapid and constant rate remarkably parallel to that of those which had remained intact during the first 20 days.

There appears to be a tendency for the outgrowth to be most rapid during the period between the twentieth and eightieth day following transplantation. During this time the diameter of the pigmented area increased from the original 2 mm. to a size somewhere between 4 and 6 mm., and hence it must have extended over the margins of the original defect in the skin. In most cases it was not possible grossly to determine whether the removal of the skin and subsequent regeneration in the area surrounding the transplant affected the outgrowth of the pigmented epidermis in this area. Since recognizable outgrowth did not occur until twenty days after grafting, regenerative processes of the epidermis and dermis over the denuded area had occurred by this time, so that a definite demarcation between the original defect and the surrounding skin did no longer exist at this time.

After about eighty days the growth rate usually began to decrease, but the outgrowth continued at a decreasing rate for as long as two hundred and eighty days after grafting. In two cases where the pigmented areas were oval in shape, growth in the largest diameter ceased at about one hundred and sixty days, but the width of the area increased slightly during the following one hundred days of observation. In Sale's experiments no data were given concerning the duration of the period of outgrowth.

As far as the direction of the outgrowth is concerned, it was of a fairly uniform character, roughly symmetrically surrounding the original graft. However, the margins of the zone of outgrowth often presented a frayed appearance, due to fine pigmented processes which appeared to push ahead of the main outgrowth. These processes did not seem to be oriented in any particular way with reference to the adjacent blood vessels, scar tissue, or neighboring pigmented areas except in one case, described below, in which outgrowth was less rapid for a time in the direction toward a neighboring area of normal brown skin.

The depth of pigmentation in the zone of outgrowth was usually of the same degree as in the original skin used for transplantation; occasionally, however, it appeared somewhat lighter, possibly due to the fine processes mentioned, between which there were strips of non-pigmented skin. The color of the outgrowth always corresponded to that of the original graft, whether the latter was black, gray or brown. We were unable to find any definite correlation between rate of outgrowth and color of the grafted piece. With one exception described below, all appeared to grow equally well. One of the best growing cases was a graft of gray epidermis, which had increased in size between the final two observations.

In this series we were not specifically concerned with the problem as to the possible effect of the amount of pigmentation or of the lack of it in the whole animal on the rate of outgrowth from the graft. In the majority of the animals used the area of pigmented skin was larger than the area of white skin, taking the body as a whole, but in one instance we used an animal that was almost entirely white except for one ear, which had a rather light brown color. From the skin of this ear two grafts were made to the white ear of the same guinea pig, and it was found that the diameters of the grafted areas increased 2 and 3 mm. respectively during a period of two hundred and forty days; whereas the average increase for the rest of the animals observed during this length of time was about 6 mm. In this single instance our results appear to confirm, perhaps, those of Carnot and Deflandre, who found that pigmented grafts extend more rapidly on animals showing a higher degree of total pigmentation. In our animals, however, the pigmentation of the one brown ear was not intense, a fact which in itself may be sufficient to explain the slow outgrowth in this case, rather than the deficiency in total body pigmentation.

Where hair was present in the area into which pigmented epidermis extended, it remained unpigmented during the period of observation. This would indicate that the pigmented skin invades surface epithelium only and does not readily extend into the hair follicles. While we concede the possibility that this may ultimately occur, it evidently requires a longer period of time than that during which our transplants were under observation.

Multiple Autogenous Transplants of Pigmented Skin to Defects in Unpigmented Skin

Double and triple grafts were made in order to obtain additional proof that the original white skin surrounding the defect becomes invaded by pigmented epithelium of the graft. When two pigmented grafts separated by an intervening area of normal white skin become connected, it is necessary to conclude that this white skin must have been invaded by the pigmented epidermis. Five double transplants and one triple transplant were made. They were separated in most instances by intact white skin varying in width from 1 to 4 mm.; but in one case both grafts were placed 2 mm. apart in the same defect.

The double grafts behaved exactly like the single ones, in that outgrowth of pigmented epidermis occurred. In two cases, where the grafts were fairly close together, fusion occurred during the period of observation. In one of these, in which the grafts were separated by 1 mm. of intact white skin, they had grown together after thirty-seven days, forming an elongated dark patch from which processes extended radially to the margin of the ear on one side, and medially to a neighboring area of normal black skin. In the case of the triple transplant two of the pigmented areas, both in the same defect, had joined by thirty days; and by sixty days all three grafts had fused across 2 mm. of intervening white skin to form a single lobated area of black epidermis. In cases where fusion did not occur the grafts had been either placed too far apart for fusion during the period of observation, or, in one case already described, the rate of outgrowth was unusually slow.

In addition to the fusion of double transplants, invasion of normal pigmented skin by outgrowth from grafted skin was also observed. In one case already mentioned where the black graft was placed at a distance of about 2.5 mm. from a normal area of brown skin, outgrowth occurred toward this brown area, although less rapidly than away from it. The outgrowth met the autochthonous pigmented margin after 95 days, and by 200 days had definitely invaded it for a distance of 2 mm. along a border of 8 mm. The black margin of the outgrowth could be clearly seen extending into the lighter brown skin. Such an observation was possible because of the difference in the character of the pigment in the autochthonous and in the transplanted area. It would have escaped detection if grafts indistinguishable in pigmentation became connected, or when a black graft joined normal black epithelium, as occurred in two cases.

In another experiment two defects, 6 mm. apart, were made in a wedge-shaped strip of white skin separating two large gray-brown areas. The smaller medial defect reached the pigmented skin on either side, while a very narrow margin of white skin was left on either side of the lateral defect. In the lateral defect a small piece of pigmented skin was placed, while the medial defect was left to heal without a graft. After a period of 56 days had passed the medial ungrafted defect was filled in by pigmented epidermis, and during a period of 74 days the outgrowth from the small graft in the lateral defect had extended across the two narrow white margins on either side. Accurate measurements of this original white zone were not made, but later observations at least indicated that the whole zone was being invaded from the adjacent pigmented surfaces, a process which was most marked along the lateral margin of the ear, at a distance of several millimeters from the graft. This suggests that in some cases the normal balance between pigmented and unpigmented epidermis may be lost. The character of the pigmented epithelium of this ear was somewhat different from that of the other animals used, being a rather uneven gray-brown, not unlike the margins of some of the outgrowths of grafted brown skin.

Discussion

We notice following transplantations a change in the equilibrium between adjoining types of epidermis which differ in their pigmentation. When, under the influence of such tissue disturbances, a shift in the balance between two neighboring types of epidermis takes place, the pigmented tissue seems to dominate over the unpigmented. However, we found indications that such an imbalance may also occur between neighboring types of epidermis which are both pigmented, but in which the kind and quantity of the pigment differs. It is conceivable that changes similar to those we have described may take place even between two adjacent areas of black skin, but such changes would not become manifest. In our case we observed movements in autochthonous epidermis under the influence of a wound in close proximity to this skin. As to the causes of these movements in epidermis, they must be distinguished from those of ordinary regeneration in which the growth takes place into a defect. Apparently the equilibrium between different types of epidermis is a very labile one which can readily be disturbed by various interferences.

Summary and Conclusions

If white skin is autotransplanted into a defect in pigmented skin in the ear of the guinea pig, the pigmented epidermis gradually invades the white. At the same time some scaling takes place in the white skin, and in the end the white epithelium becomes replaced by pigmented epithelium. This process could be completed in a minimum of twenty-four days, but in other cases it took as long as two hundred days. In the end the area where the white epidermis was replaced by pigmented epidermis was thickened and showed deeper pigmentation. There was no difference noticeable in a limited number of cases in the rapidity of invasion of the white transplant by black, gray, or brown epidermis. Rate of invasion does not therefore seem to depend on the intensity or kind of pigmentation of the surrounding host epidermis.

If pigmented skin is autotransplanted into a defect in white skin in the ear of the guinea pig, an outgrowth of the pigmented epidermis into white autochthonous skin takes place. As an initial process pigmented as well as white epithelium grows into the defect, but this is followed by the growth of pigmented into the original white skin. Definite outgrowth was first observed at about twenty days following transplantation and the outgrowth became most rapid between twenty and eighty days. Following this period outgrowth usually continued at a decreasing rate but could still be observed as late as after two hundred and seventy days. The original hair of the invaded white epidermis remained unpigmented during this process, indicating that the pigmented cells invaded the surface epithelium first and had not yet penetrated into the hair follicle epithelium at the time when the experiments were concluded. There was no correlation observed between color and intensity of pigmentation of the transplanted epidermis and the rapidity of its outgrowth into the surrounding white skin.

In case multiple transplantations were made, it could be observed that the two neighboring pigmented transplants gradually invaded the white skin separating them and thus a junction between the two pigmented transplants was accomplished. This observation provides an additional proof for the conclusion that it is actually the original white host epithelium which is invaded and replaced by the transplanted pigmented epithelium.

In accordance with these findings, we could observe a junction taking place between a black transplant and an area of brown autochthonous epidermis from which the transplant had originally been separated by an adjoining area of white skin. In this case it could be noted that the black skin invaded not only the white epidermis, but also the brown pigmented epidermis; and it may therefore be concluded that not only white skin may be invaded by black pigmented skin, but also pigmented skin may be so invaded.

In one case it could be observed that originally white skin became partly invaded by adjoining pigmented skin of the host. Thus the pigment pattern of the skin became changed, apparently spontaneously. However, it is possible that transplantation of black skin into white skin at a short distance from the area where the first-named change took place may have been responsible for this disturbance in the equilibrium between adjoining pigmented and unpigmented autochthonous epithelium.

BIBLIOGRAPHY

CARNOT, PAUL, AND CL. DEFLANDRE, 1896. Persistence de la pigmentation dans les greffes épidermiques. *Compt. rend. Soc. Biol.*, **48**: 178.

HERRICK, EARL H., 1932. Mechanism of movement of epidermis, especially its melanophores, in wound healing and behavior of skin grafts in frog tadpoles. *Biol. Bull.*, **63**: 271.

LOEB, LEO, 1897. Über Transplantation von weisser Haut auf einen Defekt in schwarzer Haut und umgekehrt am Ohr des Meerschweinchens. *Arch. f. Entwick. Mech.*, **6**: 1.

RAND, HERBERT W., AND MADELENE E. PIERCE, 1932. Skin grafting in frog tadpoles: local specificity of skin and behavior of epidermis. *Jour. Exper. Zoöl.*, **62**: 125.

SALE, LLEWELLYN, 1913. Autoplastic and homoeoplastic transplantation of pigmented skin in guinea pigs. *Arch. f. Entwick. Mech.*, **37**: 248.

SEELIG, M. G., 1913. Homoöplastic and autoplastic transplantation of unpigmented skin in guinea pigs. *Arch. f. Entwick. Mech.*, **37**: 259.

SEEVERS, C. H., AND DONALD A. SPENCER, 1932. Autoplastic transplantation of guinea-pig skin between regions with different characters. *Am. Nat.*, **66**: 183.

RECOVERY CHANGES IN TRANSPLANTED ANTERIOR PITUITARY CELLS STRATIFIED IN THE ULTRA-CENTRIFUGE[1]

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With the thought of possibly getting some light on the beginnings of malignancy, by upsetting the normal conditions within cells and observing the subsequent effects if any on the growth and functions of such cells, the authors subjected various rat tissues to intense centrifugalization and then implanted them in young rats. Inasmuch as they have been particularly interested in a type of vacuolation that appears in the basophile cells of the anterior pituitary body following the implantation of carcinoma (Guyer and Claus, 1932, 1933, 1934, 1935), they devoted special attention to the transplants of centrifuged pituitaries, and the present paper is a report of their observations on this material.

To avoid complications which might arise from the use of anæsthetics the 39 young adult rats from which the pituitaries were to be taken were killed by a blow on the head, the glands were rapidly dissected out and placed in isotonic Locke's solution. A small bit of each pituitary was used as a normal implant into a control rat, or in a few cases, for sectioning; the remainder was placed in the isotonic solution in the metal rotor of a Beams air-driven ultracentrifuge and rotated at a speed which produced a displacement pull of about 400,000 times that of gravity. The pituitaries from 20 rats were rotated for 20 minutes, then removed from the rotor, a bit was retained for cytological inspection and the rest was immediately implanted subcutaneously into twenty young rats of between 65 and 90 grams weight, one piece of pituitary to each rat. In a later experiment the pituitaries from 19 rats were removed and centrifuged, using exactly the same procedure as in the earlier experiment except that the material was centrifuged an hour before implanting it in the young rats.

The effect of an hour of such centrifugalization is shown in Fig. 5. The materials of the cell are almost wholly stratified. After 20 minutes of rotation in the centrifuge the stratification is very evident but not complete. As a result of such centrifuging the Golgi apparatus

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becomes compacted into what seems to be a single liquid mass and comes to occupy the centripetal side of the cell; that is, it is among the lightest of the cell contents. The nucleus is displaced toward the opposite or centrifugal side of the cell.

In basophile cells the nucleus seemed generally to be forced up against the cell wall but in the oxyphile cells a layer of deeply staining oxyphile granules often intervened between them. The oxyphile granules, indeed, seemed to be the heaviest particles in the cell and after prolonged rotation they became massed at the centrifugal side of the cell with the nucleus forced into their midst on the side toward the cell center.

Examination of material rotated for 20 minutes shows, however, that the displacement of different cell contents is at different rates of speed and that stratification in one kind of cell may be induced much more rapidly than in another, thus indicating different degrees of viscosity. The oxyphile cells of the anterior pituitary, for example, respond more slowly than do the basophiles and with the less prolonged centrifugalization one gets the impression at first that the nucleus is coming to lie at the centripetal side of the cell. This condition is temporary, however, and is probably due to the fact that the heavier oxyphile granules are being concentrated into the center and opposite side of the cell, temporarily displacing the nucleus toward the centripetal side. Eventually it is forced to the centrifugal side and, as just described, becomes more or less imbedded in the mass of oxyphile granules.

From their respective behaviors under centrifuging it is evident that the oxyphile granules found in pituitary cells are heavier than the basophile granules. The centrifuge also reveals that even in the large so-called basophile cells there are considerable numbers of oxyphile granules, since in such cells when differentially stained a thin zone of acid-stained particles is visible.

The chromatin content of the nucleus is densely massed at the centrifugal side although strands of an achromatic substance, presumably linin, remain stretched across the nuclear body, giving the appearance of being attached to the opposite nuclear wall. Evidence of this is seen in the nuclei shown in Fig. 5. Inasmuch as this condition has been rather fully discussed and depicted in a recent paper by Dornfeld (1936) in connection with cells of the adrenal gland, based in part on material from the same rats as were used by us in the present study, further comment on the nature of the nuclear displacements is unnecessary; the conditions seem to be similar in all respects.

The implanted tissue which had been centrifuged for 20 minutes was removed and fixed for cytological study after different periods of growth, as follows:

Tissue	planted	24	hours,	from	1 rat.
“	“	48	“	“	1 rat.
“	“	4	days,	“	1 rat.
“	“	7	“	“	4 rats.
“	“	14	“	“	5 rats.
“	“	21	“	“	4 rats.
“	“	28	“	“	1 rat.
Implants did not grow in					3 rats.
					20 rats.

The most striking fact to be noted in the transplanted pituitary cells was the rapidity with which those which survived resumed relatively normal appearance. At the end of 24 and of 48 hours respectively the removed implants showed the presence of much necrotic tissue and a heavy invasion of polymorphs, but this was true of control transplants as well; hence it cannot be attributed to the centrifuging. As soon as the vascularization of the transplanted tissue became well established this condition rapidly cleared up and the cells, particularly those near blood vessels, began to look normal. The last thing to be restored to its characteristic normal appearance was the Golgi apparatus. This structure seems to be very sensitive to changes in general, for even in normal, non-centrifuged anterior lobe material it tended to become clumped and pyknotic looking. The return of the nucleus to the central region of the cell and the redistribution of the various cell granules seems to follow promptly the cessation of centrifuging. In another set of experiments (ms. in press) carried on in this laboratory by Halcyon W. Hellbaum on centrifuged snake thyroid, the brief interval between the start of tissue fixation and its completion seemed to be sufficient for the nucleus to make an appreciable return toward the cell center; for with centrifuged thyroid tissues, in the halves of thyroid glands that were fixed in rapidly penetrating fluid such as Bouin's, the nucleus was noticeably nearer the centrifugal margin of the cell than in the other halves of the same glands fixed in more slowly penetrating fluids such as Champy's (bichromate-osmic-chromic acid) mixture. This would indicate that cytoplasmic elasticity enters as a significant factor.

Immediately and for some time after centrifuging, the Golgi complex seems to be but a dense rounded drop of material at the centripetal side of the cell. It comes back to its network-like configuration very slowly, attaining it again only at the end of two or three weeks. It first loses its dense appearance by breaking up into a multitude of fine droplets, so small often as to give almost a powdery appearance. As time goes on the droplets become larger, apparently by more or less coalescence. In pituitary tissue which had been centrifuged for 20 minutes and then removed after an implantation of 14 days, the Golgi complex is usually back in the neighborhood of the nucleus though it is commonly still in a divided state of droplet-like particles. These are grouped more on one side of the nucleus although they may more or less surround it. A few examples of a return to the network type of Golgi body are to be seen in some of the basophile cells; still more occur in the oxyphiles.

Even at the end of three weeks a considerable amount of granular Golgi material is still in evidence although the prevailing Golgi apparatus is of the network type. However, this slow return of the Golgi complex to the condition of a network is probably not determined by the fact of earlier centrifugalization since control, non-centrifuged pituitary recovered after three weeks of transplantation may similarly show a considerable degree of granulation.

By the end of the fourth week the transplants, both centrifuged and non-centrifuged, seem to be rather generally on the decline, and possibly final disintegration is approaching. This is particularly true of the basophile cells; their Golgi material shows much granulation or droplet formation.

Figure 6 is a composite picture of two sections of recovered anterior pituitary which had been centrifuged for 20 minutes and then implanted for seven days. That the tissue is anterior pituitary gland is evident. Certain of the basophiles (*b*) and the oxyphiles (*o*) have been lettered to identify them more readily.

Of the 19 rat pituitaries which were centrifuged for an hour before implanting, only 2 survived. Operations at the end of two weeks revealed that 2 had been replaced by pus sacks and the remaining 15 had been resorbed. In the 2 bits of tissue which were alive at the end of two weeks the basophiles and oxyphiles seemed to be in a thriving condition. They did not differ in any noticeable particular from similar implants of the 20-minute series.

In one of the anterior pituitaries which had been centrifuged 20 minutes and then recovered after three weeks of implantation, a peculiar pituitary cyst was encountered (Figs. 3, 4). It strongly suggested the so-called colloid follicles so characteristic of the thyroid. Cysts of supposedly colloid-secreting cells are occasionally found in the pituitary glands of normal animals including man. The striking thing about the cyst in our transplant was the fact that it had apparently been stimulated to increased activity. The lumen was filled with a vacuolated, gluey looking mass and the secreting cells had become columnar instead of cuboidal, much enlarged, and in many cases they looked like goblet cells. This raised the question of whether they were typical colloid cells or were mucus-secreting cells, and also of just what the difference between a colloid and a mucous secretion is. With the Mallory triple stain the secretion gave the typical blue colloid picture but when Mayer's muci-carmine stain was used the picture was just as typically that of mucus-secreting cells with various of the cyst-wall cells staining as deeply as do characteristic goblet cells. This was true even in sections which had been stained in Mallory's triple stain, and later destained, and stained again in Mayer's muci-carmine. Thyroid follicles, however, subjected to the same treatment did not give the muci-carmine response. The pituitary cyst from a control animal (Figs. 1 and 2), on the other hand, did give the typical muci-carmine reaction.

In Figs. 1 and 2 photographs of a cyst from the anterior pituitary of a control and supposedly normal rat are shown at different magnifications. While the contents of the cyst lumens seem the same in the control and in the experimental animals, the secreting cells are very different in appearance in the two. Those from the control (Figs. 1 and 2) are cuboidal, those from the centrifuged and transplanted pituitary are conspicuously columnar in appearance. Both sets are ciliated. We have thoroughly canvassed the possibility that such appearances as those shown in our photographs (Figs. 1 to 4) might be due merely to striations in the mucin or in the shrinkage space between the mucoid mass and the epithelium lining, but careful inspection of these and other adjoining sections, both by ourselves and others in our laboratory accustomed to making cytological observation under high

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EXPLANATION OF PLATE I

1. Photomicrograph of a colloid or mucoid cyst in the anterior pituitary of a normal rat, showing the ciliated epithelial lining of the cyst wall and the contents of the lumen. × 376.

2. Photomicrograph of a part of the foregoing cyst, under higher magnification. The cuboidal, ciliated epithelial cells are obvious. × 848.

3. Photomicrograph of a mucoid cyst in anterior pituitary material that had been centrifuged in an ultracentrifuge for 20 minutes, then grown in a young rat for three weeks, after which it was removed and sectioned. The epithelium which lines the cyst wall consists of very active, columnar cells, mostly ciliated, though some have the appearance of being goblet cells. × 376.

4. Photomicrograph of a part of the cyst shown in Fig. 3. Occasional smaller cells are observable between the bases of the columnar cells and the basement membrane. × 848.

5. Photomicrograph of cells from a rat hypophysis centrifuged in an ultracentrifuge for one hour. The deeply stained material of the nuclei is the chromatin substance forced to the centrifugal side of the nucleus. In some of the nuclei strands of achromatic material, presumably linin, are visible stretching across the nuclear body, because still attached, seemingly, to the opposite nuclear wall. × 848.

6. Photomicrographs from two slightly different regions of a bit of anterior pituitary gland grown 7 days in a young rat after having been centrifuged for 20 minutes. Typical basophile (*b*) and oxyphile (*o*) cells are to be seen. × 848.

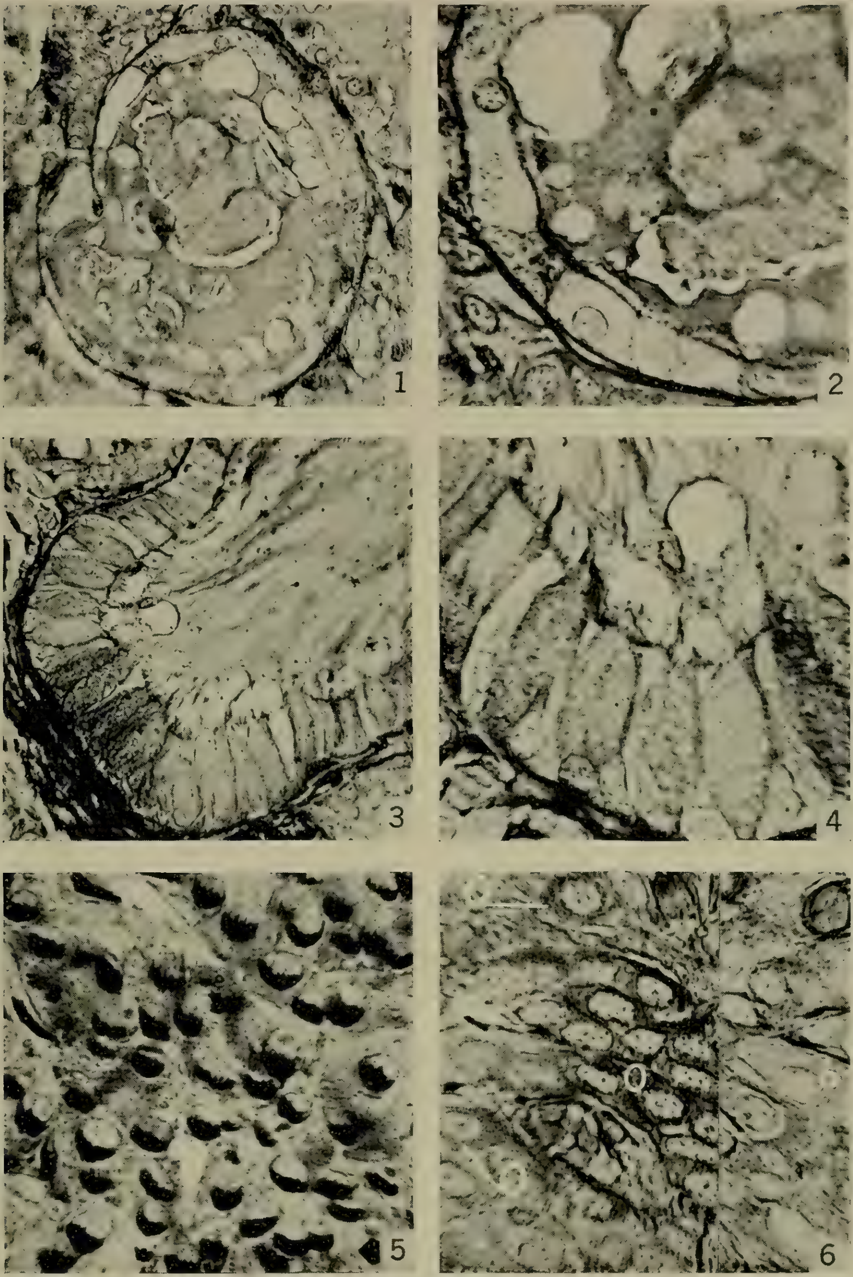

Plate I

powered lenses, convinces us that the objects in question are not artefacts but cilia.

Whether the goblet-like cells of the transplant are transformed ciliated cells we are unable to say. And whether or not the wall cells of these cyst formations are to be regarded as unusual differentiations of pituitary tissue, as residual cells from an earlier epithelium, or migrant elements from the naso-pharyngeal region, we cannot say. The thing of chief interest in our present study is the enhanced secretory activity shown by the centrifuged cells.

In the literature dealing with such hypophyseal cysts the nearest approach to the types of cell shown in our preparations is that depicted by Rasmussen (1929) from sections of human hypophyses. Several of his pictures from human material look almost exactly like what we find in implanted centrifuged pituitary tissue from rats.

Summary

Anterior pituitary tissue which has had its cell contents stratified through 20 to 60 minutes of rotation in an ultra-centrifuge with a displacement pull of about 400,000 times that of gravity, returns in many instances to normal appearance, displaying the characteristic basophile and oxyphile cells, when transplanted into young rats. An hour seems to be nearly the maximum time such tissues can be thus rotated and remain viable. Nuclei forced completely to one side of the cell rapidly resume their normal location near the cell center, with their displaced chromatin contents apparently restored to normal distribution. The Golgi apparatus, concentrated by the rotation into a liquid drop at the centripetal side of the cell, is the last part of the cell complex to resume its characteristic appearance, which is that of a network. A mucoid cyst with hypertrophied and extremely active epithelial wall cells, most of them ciliated, is described and compared with the type of cyst that is occasionally found in the normal pituitary.

LITERATURE CITED

Dornfeld, Ernst J., 1936. Nuclear and cytoplasmic phenomena in the centrifuged adrenal gland of the albino rat. *Anat. Rec.*, **65**: 403.

Guyer, M. F., and Pearl E. Claus, 1932. Cell changes in the anterior lobe of the pituitary following cancer transplantation in rats. *Anat. Rec.*, **52**: 225.

Guyer, M. F., and Pearl E. Claus, 1933. Cellular constituents of the anterior hypophysis after uterine implants of carcinoma in rats. *Anat. Rec.*, **56**: 373.

Guyer, M. F., and Pearl E. Claus, 1934. The Golgi apparatus in relation to vacuolation in the basophiles of the anterior pituitary of castrate and of cancerous rats. *Anat. Rec.*, **61**: 57.

Guyer, M. F., and Pearl E. Claus, 1935. Vacuolation in the anterior pituitary of castrated and of thyroidectomized rats. *Anat. Rec.*, **61**: (suppl.) 21.

Rasmussen, A. T., 1929. Ciliated epithelium and mucus-secreting cells in the human hypophysis. *Anat. Rec.*, **41**: 273.

COMPONENTS OF THE MITOTIC SPINDLE WITH ESPECIAL REFERENCE TO THE CHROMOSOMAL AND INTERZONAL FIBERS IN THE ACRIDIDÆ

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INTRODUCTION

While my own studies are concerned with specific components of the mitotic spindle, I believe the structure should be considered as a whole and I shall endeavor, accordingly, to summarize our knowledge of certain points.[2]

Centrioles

The beautiful demonstration of these structures in pole cells of *Drosophila* eggs which complete their development (Huettner and Rabinowitz, method published in 1933) must validate the reports of these structures in fixed material even for the skeptical. The following conclusions in regard to them seem to be warranted by careful work.

Physical Characteristics.—They are visible in living cells where they undergo vibratory motion and give evidence of being more rigid than the surrounding cytoplasm. They are usually spherical but their ability to vary in shape is well shown in the tree-crickets (Johnson, 1931) where the centrioles are minute spheres in the spermatogonia, comparatively large V's in the first spermatocytes and specialized rods in the second spermatocytes. In the protozoan, *Barbulanympha,* as shown by Cleveland et al. (1934), they are elongate, flexible rods of relatively large size. In all cases, they stain with iron-hæmatoxylin but in *Ascaris,* at least, they do not give the Feulgen nucleal reaction (Carothers, unpublished).

Position.—Centrioles are located usually just outside the nuclear membrane but they may be near the cell membrane, as in the first spermatocytes of the tree-crickets, or intranuclear, as in certain of the Protista and in the male germ cells of *Ascaris*. During mitosis they come to lie on opposite sides of the nucleus, 180 degrees apart.

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[1] This work was begun at the Zoölogical Laboratory of the University of Pennsylvania.

[2] A much fuller discussion of this subject is to be found in the third edition of E. B. Wilson's excellent book, "The Cell in Development and Heredity." Macmillan, 1925.

Origin.—Normally, they are self-perpetuating structures which undergo division at each mitosis. In addition, a so-called de novo origin seems to have been demonstrated in certain instances. Wilson (1901) showed that in artificial parthenogenesis in sea-urchin eggs numerous cytasters each with a centriole-like body arise in one egg. These "central bodies" and their cytasters divide and give rise to typical amphiasters which later are separated by cleavage furrows. Even more impressive is the fact that cytasters may unite with a nuclear aster to form a multipolar spindle with irregular distribution of the chromosomes to all poles followed by multipolar cleavage. A "de novo" origin may occur also in partheno-produced grasshoppers (Slifer and King, unpublished). Centrioles appear to be lacking during the maturation divisions of the eggs, yet the early cleavage cells of embryos arising from unfertilized eggs have typical centrioles. Here, however, there is the possibility of the survival of the egg centriole even if it does not function during meiosis. In the case of artificial parthenogenesis I would suggest that certain of the formed components of the cytoplasm may be able under especial circumstances to take on the function of centrioles.

Distribution.—Centrioles are present generally in the Metazoa. An exception is the oöcytes in which they may either be lacking as in *Ascaris* or present as in *Crepidula* where Conklin (1902) showed that a division center is formed with each pronucleus. They are lacking in the higher plants, while in the lower plants (cycads, *Ginkgo*, bryophytes and pteridophytes) they are absent in the somatic cells and those of the early germ-line but appear as typical centrioles with asters at a definite stage in the life cycle, namely, the last divisions which give rise to the male sex-cells.

Function.—Aside from their prominent part as division centers in mitosis, centrioles are associated with motility as the blepharoplasts which form the locomotor apparatus of the sperm cells of the lower plants, the flagellate Protozoa and the flagellate cells of sponges as well as the sperm cells of animals. In the case of *Barbulanympha*, referred to previously, and spermatids, the centriole divides and the proximal one takes no part in forming the locomotor apparatus. This suggests, as has been pointed out by other workers, that centrioles may contain two components only one of which functions as a blepharoplast.

Centrosomes

This term denotes the regions of specialized cytoplasm surrounding the centrioles about which the constituents are arranged concentrically.

Near the centrioles the groundwork is homogeneous or finely granular, and, in well-fixed material, the astral rays are seen to traverse it. Centrosomes vary greatly in size and complexity in different organisms and may be entirely absent as in some of the hypermastigotes (Cleveland et al., 1934).

Astral Radiations and Central or Continuous Spindle Fibers

Perhaps the most significant work to date on these structures is that just mentioned of Cleveland and his collaborators. They report, on what they consider absolutely conclusive evidence derived from both living and fixed material, that astral rays and central spindle fibers arise from the centrioles. Furthermore, and even more important, they state: "The fibers of the achromatic figure . . . arise from the opposite ends of the same organelles (centrioles [3]) as the flagella; and they are like flagella in individuality, size, and appearance both in living and stained organisms; but, unlike flagella, they are not able to move independently." Flagellates in roaches undergoing ecdysis form intracytoplasmic bundles of flagella; these are used as a basis of comparison. Lucas (1932) described a ciliature anlagen for *Cyathodinium* in which the cilia are formed intracytoplasmically, before they are evaginated upon the surface. These two cases show clearly that definite, thread-like structures possessing elasticity and tenacity can be differentiated in the cytoplasm; hence, there is little reason to doubt that spindle fibers exist in living cells essentially as they appear in well-fixed material.

Another well-established and critical fact, already mentioned, is that compound achromatic figures may be formed by the union of asters of different origin, due either to double fertilization where the two sperm may come from different males or to artificial fertilization, where cytasters may join with a normal amphiaster. Incidentally, tripolar spindles are the strongest evidence against the idea that the mitotic figure is due to a polarized field since lines of force form only between plus and minus poles. Another fact which does not fit with such a hypothesis is that in normal mitosis the rays from the two asters cross at the equator, whereas lines of force in a bipolar field are continuous from pole to pole. Both of these facts were recognized by early workers. I mention them now because several recent observers ignore them.

Interzonal Fibers

These are the fibers which connect the distal ends of daughter chromosomes for a brief time in anaphase. (See p. 482.)

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[3] The parenthetical expression is mine.

Chromosomal Fibers (Half-spindle Fibers)

These structures pass from a fixed locus on each chromatid to the poles. The shape of the chromosome is correlated with the position of these loci.

Mitochondria

Perhaps these elements should be mentioned since they surround the spindle densely, especially in insect meiosis (Bowen, 1920), and probably prevent the fibers themselves from being seen in living cells.

TECHNIQUE AND NOMENCLATURE

The material was fixed in either strong Flemming or my own fixative, the formula for which may be found in Dr. McClung's *Microscopical Technique.* The stains used were Flemming's tri-color and Heidenhain's iron-hæmatoxylin. Most of the material was stained prior to 1920.

In regard to terminology, I shall use the phrases "chromosomal fibers" or "chromosomal processes" interchangeably to designate the structures which pass from the chromosomes to the poles in preference to the expression "half spindle fibers" recently revived by Schrader, and I hope to show that the term "point of fiber insertion" should be changed to "point of origin."

OBSERVATIONS

My observations concern, chiefly, the chromosomal and interzonal fibers. The X-chromosomes of short-horned grasshoppers lend themselves very readily to the study of chromosomal fibers for two reasons:

1. They are a step in advance of the other chromosomes in the first spermatocytes. Consequently, they reach the physical condition of euchromosomes in metaphase at late diakinesis and at the actual metaphase are becoming diffuse for the following telophase.

2. The point of origin of spindle fibers varies in different species but is constant, typically, for the individuals of a given species; occasional exceptions to this rule occur, however. For example, most species have telomitic X-chromosomes but certain species of *Trimerotropis* and allied genera have atelomitic X-chromosomes with rare individuals which not only have both types but intermediate forms as well. Plate I, Fig. 1 shows partial complexes from three adjoining first spermatocyte metaphases of such an individual of *T. coquilletti.* At

C the X-chromosome (solid black) is telomitic, at *B* it is atelomitic with a process from each chromatid while at *A,* obviously, it is in a dilemma. The process for a terminal fiber is present, at the lower right end, the median processes are evident above, and in addition the opposite end has two subterminals going to opposite poles. The chromosome is flattened and distorted as though subjected to conflicting stresses. Eventually such an X-chromosome will pass to one pole or the other without division, due apparently to the retraction of some of the processes. A count of 115 first spermatocyte metaphases gives the X-chromosome telomitic in 40, atelomitic in 50 and showing evidence of a conflict in 25.

Perhaps the reader should be reminded that in the grasshoppers each egg which produces a male has only one sex-chromosome at the time of fertilization and that is contributed by the mother. All of these variations, therefore, have arisen in this individual. A change from telomitic to atelomitic or vice versa might be due to inversion of a segment of the chromosome. This, however, would not cause a change in the number of fibers. Conceivably, the extra fibers might be acquired by translocations involving spindle fiber loci from other members of the complex but in that case a cell in which the X-chromosome has fibers arising from three points should show two of the other chromosomes behaving eccentrically because of the loss. Such abnormalities are not present.

Naturally a study of these multiple spindle fibers and of shifts in position of the fibers lead to a consideration of the origin and nature of the fibers which pass from the chromosomes to the poles.

In 1917 I reported (p. 459) and figured (Plate 7, Fig. 45) a single X-chromosome in *T. fallax* which showed several distinct fibers passing towards both poles of the spindle. At that time, I simply recorded it as an unmistakable fact with no surmises as to the cause. It is reproduced as Fig. 2 of the present paper for the convenience of the reader and for comparison with other X-chromosomes which exhibit a similar phenomenon.

Figure 3 is from the same individual as Fig. 1. Note that the apex of the V is marked by a group of delicate fibers. Schrader has emphasized the existence of a like condition in other forms. Figure 4 is from *Derotmema laticinctum,* a species which usually has no atelomitics. Here, I think, the solution of the problem becomes self-evident when we compare this X-chromosome with the large tetrads in diakinesis just below it (Figs. 6 and 7). Delicate pseudopodial-like processes which contact either those from other chromosomes (Fig. 7, *A, B*) or the nuclear membrane where they form enlarged or even plate-like contacts (Fig. 8) are put forth at this stage,[4] apparently, from every chromomere except those which resemble the X-chromosome in precocity. These processes can be retracted almost instantly as is attested by the chromosomes of contiguous cells which show transitions such as are illustrated in Figs. 6, 7 and 11. This cyst was fixed, fortunately, at the moment when the cells were passing from late diakinesis to early metaphase; in a few cells the nuclear membrane had disappeared and chromosomes, only slightly smoother than the tetrad at the right in Fig. 11, were forming the equatorial plate. Short processes comparable to those on the X-chromosomes shown in Fig. 9 are evident, occasionally, on such tetrads at the spindle fiber loci. The whole testis appears to be normal so that I believe the variations shown to be the regular occurrence. In fact, tetrads of like density, as denoted by staining reaction, vary within the same nucleus in regard to the degree of contraction or extension of their processes and even the parts of a single tetrad exhibit this diversity, as may be seen in Fig. 6, where the ring-shaped tetrad has some processes long and attenuated and others short and blunt as though they had been retracted suddenly. Figure *7A* is an end view of one arm of a tetrad which illustrates the fact that the processes are sent out not only at right angles to the plane of the next division but in various directions. Club-shaped ends on processes which have severed their contacts, such as are illustrated at *7B,* suggest that external stimuli applied to the process itself may cause retraction as well as intrinsic forces which presumably usually cause their withdrawal.

Figure 9 shows two late diakinesis X-chromosomes in their actual position relative to each other. Note that they are as fully condensed as the tetrads are at metaphase. The single process on each is in the usual position for the spindle fiber of the X-chromosome in this species and in these instances is certainly of chromosomal origin. Figure 10 from the same cyst represents an X-chromosome which has maintained an end-to-end association with a tetrad up to this late stage, and I suspect such an occurrence accounts for the telomitic X-chromosomes in this species. Figure 12 is from an earlier cyst. Approximately half of the X-chromosomes are U-shaped and half rod-shaped in this stage. At first thought one might assume that such forms are the direct precursors of the V and rod-shaped X-chromosomes, respectively, of the metaphase, but this is improbable because in all species of short-horned grasshopper which I have observed, cytologically, the X-chromosomes form close U's at about this stage and a little later thick rods, regardless of whether the X-chromosomes at metaphase are telomitic or atelomitic.

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[4] To obtain these processes at their best a number of precautions are necessary. First the animal should be killed in a suitable manner such as the application of xylol to the spiracles, next the testes should be removed from the body with delicacy and care in order to avoid unnecessary shock and quickly transferred to an abundance of good fixative. Finally the stain must not be extracted too much, the processes are chromophilic but very attenuated.

With the sex-chromosomes at their maximum density during late diakinesis and beginning to become diffuse just before the metaphase and with the roughened diakinesis tetrads becoming more compact as they approach the metaphase, a brief period occurs when the two types of chromosomes present similarly roughened contours. Such a stage is shown in Figs. 13 (X-chromosome below), 14 (X-chromosome above), 15 (X-chromosome only) and 11 (tetrads). Compare also the tetrad in Fig. 14, on which corresponding processes are still evident on sister chromomeres, with the sex-chromosome from *Circotettix verruculatus* shown in Fig. 5 which has put forth a process toward both poles for each of five aggregates of chromomeres.

The foregoing observations seem sufficient to raise a strong presumption that the chromosomal fiber of the sex-chromosome is a process put forth by the chromosome itself.[5]

Let us now consider some later stages; first, the metaphase spindle, next conditions which immediately precede the metaphase, and finally the anaphase. Naturally, many of the points which I shall mention have been observed by other cytologists. Four critical features, however, seem to have escaped notice. Attention will be called to them in the following pages.

In lateral views of well-formed metaphase spindles, two kinds of fibers are evident on a morphological basis. The continuous fibers, which in the grasshoppers do not form a central spindle but are intermingled with the other spindle components, are slender, of uniform thickness and, in fixed material, somewhat wavy. The chromosomal fibers run a straighter course and are much heavier at their loci of emergence from the chromosomes, from which they taper gradually towards the centrioles,—thus, they vary in thickness throughout their length. These differences are illustrated in Fig. 18 from a spermatogonium of *Circotettix rabula* and Figs. 24 and 25 from first spermatocytes of *Trimerotropis citrina*. Convinced that the chromosomal processes pos-

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[5] At this place I should like to call attention to another point. The first three drawings in Fig. 11 are of the same tetrad in the size series from three cells. In addition to showing the transition of contour already mentioned, they are of interest on account of the characteristic spherules which mark this tetrad in late diakinesis and which probably are either secretory or excretory products. They are usually embedded in the surface chromatin but may be at a considerable distance from the parent chromosome with which they are still connected by what appear to be delicate fibrils. Later these connections may disappear, leaving some of the spherules free in the cytoplasm where for a time they continue to give the Feulgen reaction. I believe, for reasons which I hope to develop in a later paper, that they are metabolic products and not true chromatin in process of elimination.

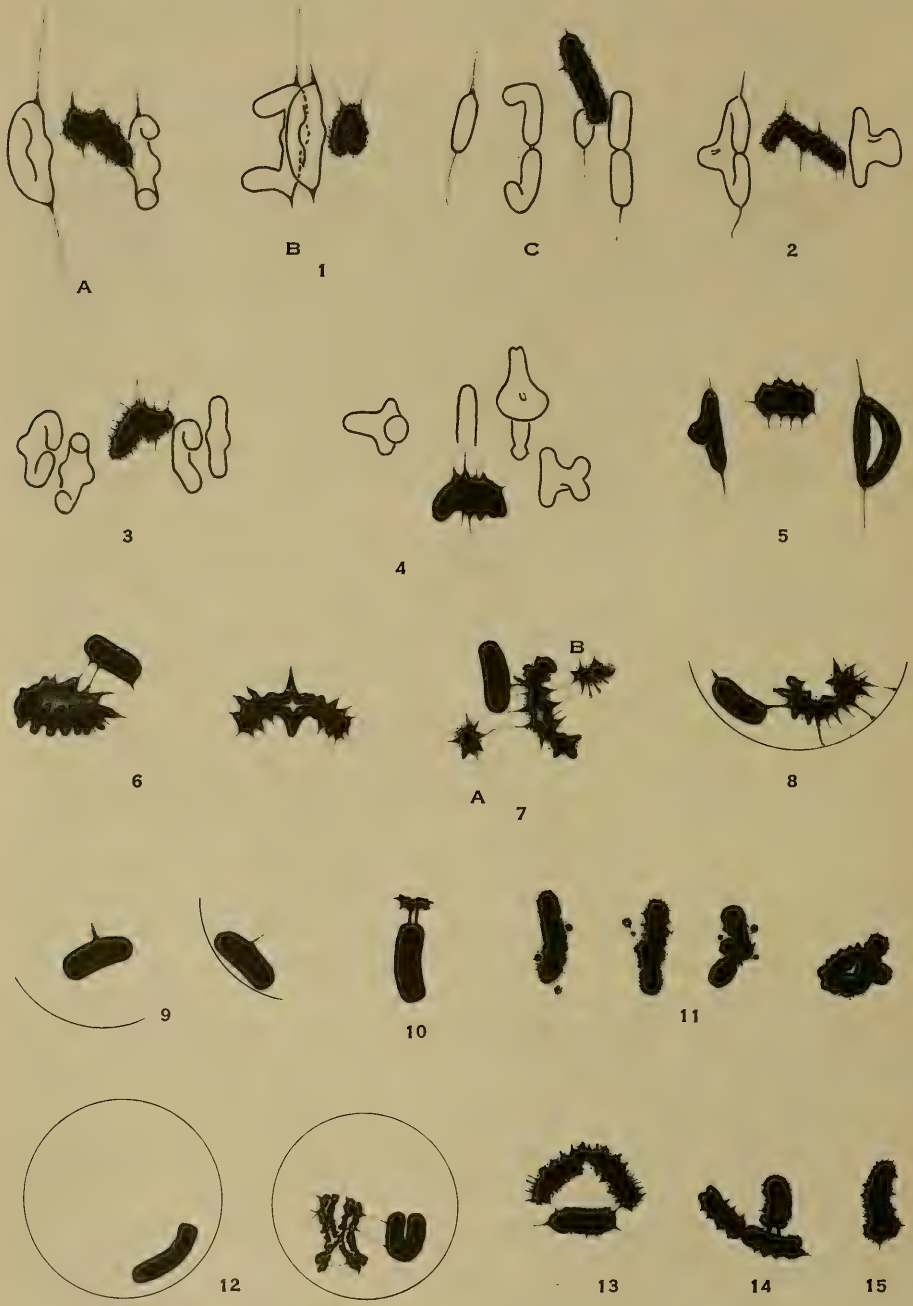
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PLATE I

sess considerable elasticity by evidence such as is represented by Figs. 23 and 29, which would be difficult to explain on any other basis, I expected them to become thicker as they became shorter in anaphase. Such, however, is not the case normally. The processes, beginning with the heavier basal parts, apparently, are reincorporated into the anaphase chromosomes as they move towards the poles, so that as they

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EXPLANATION OF PLATES

All of the figures were drawn with the aid of a camera lucida at a magnification of 2,800 diameters. They were reduced one-third in reproduction.

Explanation of Plate I

Fig. 1. Partial complexes from three contiguous first spermatocyte metaphases, lateral views. X-chromosome, solid black. At *b* it is atelomitic, at *c* telomitic and at *a* the telomitic process is evident at lower right end which is of normal thickness, the apex of an atelomitic V with two processes is indicated above while the other end is flattened and has processes towards each pole. *Trimerotropis coquilletti.*

Fig. 2. Partial complex of a first spermatocyte lateral view, *T. fallax.* Note the three prominent processes of the X-chromosome.

Fig. 3. Similar to preceding. X-chromosome with numerous fibers or processes. *T. coquilletti.*

Fig. 4. Similar to Fig. 3. *Derotmema laticinctum.* Compare this X-chromosome at metaphase with the diakinesis tetrads shown in Figs. 6, 7, and 8.

Fig. 5. Similar to above, except the tetrads also are in solid black. *Circotettix verruculatus.*

Figs. 6–15. Late first spermatocyte prophases, from *T. coquilletti,* showing transition of X-chromosome from the smooth contour characteristic for it in diakinesis and for the tetrads in metaphase (Figs. 6, 7, 8, and 12) to a roughened contour in very late diakinesis (Figs. 13, 14, 15). Note the long pseudopodial-like processes of the tetrads (Figs. 6, 7, 8) which are withdrawn at the approach of metaphase (Figs. 11, 12 and 13).

Fig. 6. X-chromosome and tetrads. Note blunt processes of the ring at left where the fibers have been retracted.

Fig. 7. Similar to Fig. 6. End views of arms of two tetrads at *a* and *b*. Processes go out in all planes and contact those of other chromosomes. Note the knobbed ends of retracting processes at *b*.

Fig. 8. Similar to above. Processes of tetrad in characteristic contact with nuclear membrane.

Fig. 9. X-chromosomes from two adjacent cells. Note the process on each and compare with spindle (chromosomal) fibers of tetrads shown in Fig. 5.

Fig. 10. X-chromosome from the same cyst which will probably be telomitic at metaphase.

Fig. 11. Tetrads from a cyst where metaphase plates are forming in some cells. The droplets associated with the three examples of one tetrad are characteristic. They may be either secretion or excretion.

Fig. 12. Nuclei from two adjacent cells, earlier diakinesis. X-chromosomes at this stage are either rod or U-shaped in about equal numbers.

Fig. 13. X-chromosome and tetrad approaching each other in roughness of outline. Very late diakinesis.

Fig. 14. The same, a trifle later stage.

Fig. 15. X-chromosome in very late diakinesis. Compare with X-chromosome in metaphase shown Fig. 1, *c*.

shorten they become more delicate. The linear order of their constituents is thus preserved and there is no loss to the chromosome. It seems to follow that the chromatids move apart through some inner mechanism and that the chromosomal processes act more as guides than as "traction fibers."

If we turn now to premetaphase conditions, we find that as early cytologists observed, the nuclear membrane disappears first on the side traversed by the separating centrioles. Some early workers also noticed that the fibers from the two asters intersect in the plane of the equator at early metaphase, but later curve inward and become part of the central spindle. A characteristic which seems to have escaped mention, however, is that in this series of actions one side is still slightly in advance of the other (see Figs. 19 and 24). This condition applies also to the arrangement of the chromosomes on the spindle. The first of the critical features mentioned above is associated with this fact. (1) In lateral views of very early metaphases when the equatorial plate is forming, the chromosomes on one side of the spindle may have typical chromosomal fibers extending from the chromosomes to the poles, while on the other side identical fibers (processes) extend from the chromosomes in the general direction of the poles, but are not yet oriented towards the centrioles. Such a case is illustrated in Fig. 16. The lower process on the small rod at the left is of especial interest because it bends outward towards the cell membrane crossing that of the larger, underlying tetrad. These structures are identical so far as they go with the unquestionable chromosomal fibers on the two tetrads at the right. Their homology with the processes on the sex-chromosomes

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Explanation of Plate II

Fig. 16. First spermatocyte metaphase, lateral view. Chromosome fibers reach the poles on one side, forming and not yet oriented on the other.

Fig. 17. Similar to above. Note separation of the processes; also, that they are thicker at point of emergence from chromosomes.

Fig. 18. Spermatogonial metaphase, lateral view, spindle very uniform. Chromosomal fibers thicker and straighter than continuous fibers. *Circotettix rabula.*

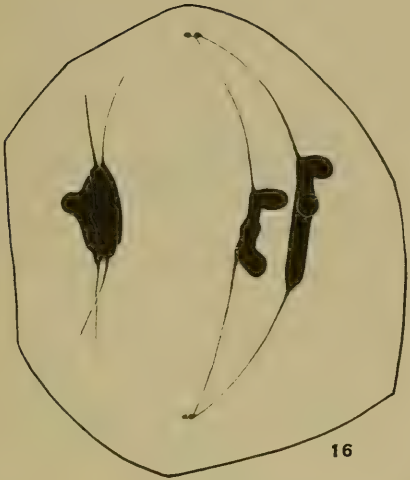
Fig. 19. Second spermatocyte, lateral view, from same individual. Processes pass straight out from chromosomes until they approach the poles, then bend abruptly towards them.

Fig. 20. Two tetrads where the point of spindle fiber origin is different for each homologue. *T. fallax.*

Fig. 21. Six tetrads in anaphase illustrating interzonal fibers. *T. fallax.*

Fig. 22. Three tetrads in metaphase showing relation of chromomeres to chromosomal fibers. *T. fallax.*

Fig. 23. Tetrad displaced in sectioning. Chromosomal process distorted but unbroken. *T. fallax.*



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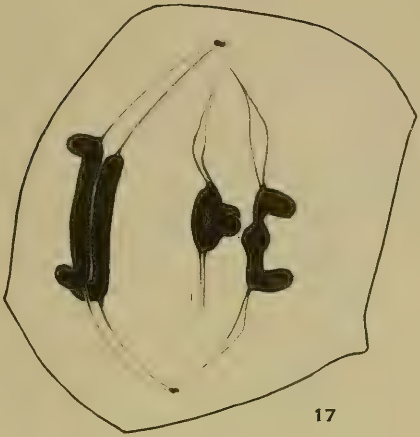

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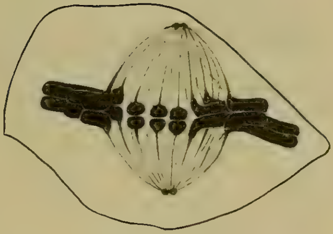

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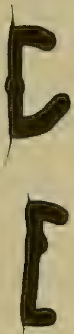

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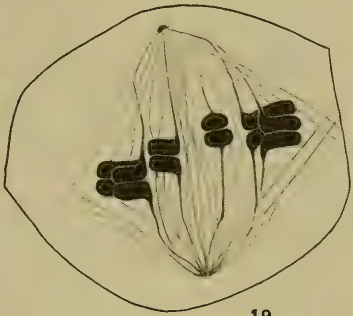

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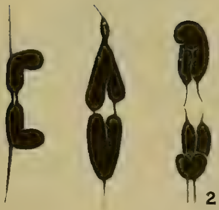

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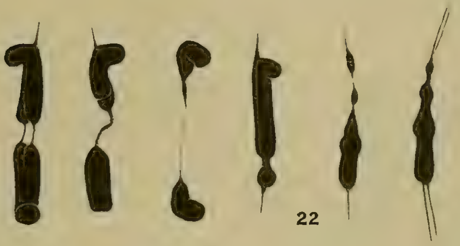

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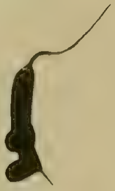

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PLATE II

shown on Plate I, especially Fig. 9, and on the tetrad at the right in Fig. 6 seems almost equally certain.

(2) The second point which I consider critical is the sudden inbending of such processes, which have tended to pass straight out from their point of origin in the chromosome, when they reach the vicinity of the centrioles (Figs. 19, 24, and 25). This abrupt inbending of the fibers lends some support to older views recently supported by Cleveland (1934, 1935*a*, 1935*b*) that fibers which lead from the chromosomes to the centrioles have a dual origin. I am convinced, however, that in the grasshoppers they are wholly chromosomal in origin; partly because, even near the poles, they are always markedly heavier than the fibers of the central spindle. See especially the dyad at the right in Fig. 29, a first spermatocyte anaphase from *Dittopternis*. Their change of direction probably indicates that they have reached a region where the centrioles exert a more active influence. Such figures occur only in early metaphase where one side of the spindle is still slightly in advance of the other. The spindle later becomes symmetrical, as shown by Fig. 18 from a spermatogonium of *C. rabula*.

(3) The third suggestive characteristic is the behavior of the chromosomal fibers, one for each chromatid, often evident on each dyad in the first spermatocyte metaphase (Figs. 17, 21, and 23).[6] They taper gradually towards the centrioles and may diverge for some distance after leaving the chromosome, later converging as they approach the centrioles (Fig. 17). This behavior is perfectly comprehensible if they are processes put forth, one by each chromatid, which extend independently, in the general direction of the poles. On such a view they might diverge considerably until they come under the influence of the centrioles, when the sudden inbending would naturally cause them to converge. It need hardly be pointed out that this behavior, as well as that described under "2," is wholly incompatible with any theory which

EXPLANATION OF PLATE III

FIGS. 24 and 25. First spermatocyte metaphases, lateral views, *T. citrina*. Individual represented in 24 killed with potassium cyanide, that in 25 with xylol. Such differences in spindle configuration characteristic for these two methods of killing.

FIG. 26. Metaphase, polar view, from same individual as Fig. 24.

FIG. 27. Similar to above, from same individual as Fig. 25. Chromosomes form a more open plate.

FIG. 28. Late anaphase, lateral view, chromosomes surrounding the poles. Same individual as Figs. 24 and 26. Spindle .69 of the length attained at metaphase when animals are killed with xylol.

FIG. 29. One pole of an anaphase. One chromosome and its fiber in plane of section. *Dittopternis turbata*.

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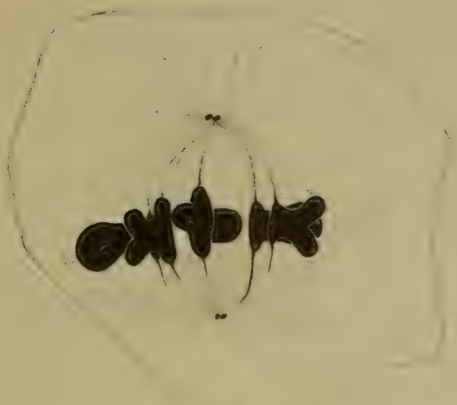
[6] Henking (1890) noted this condition in first spermatocytes of *Pyrrhocoris* and recognized it to be associated with the valence of the chromosomes.



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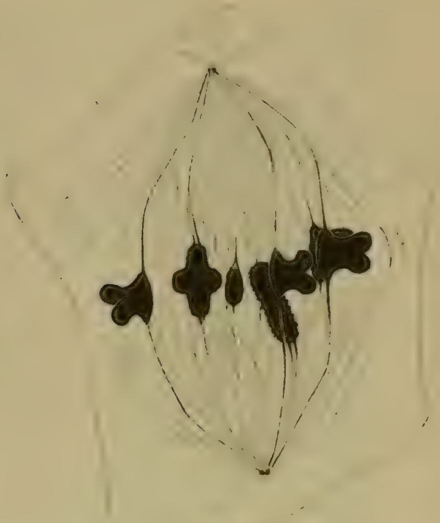

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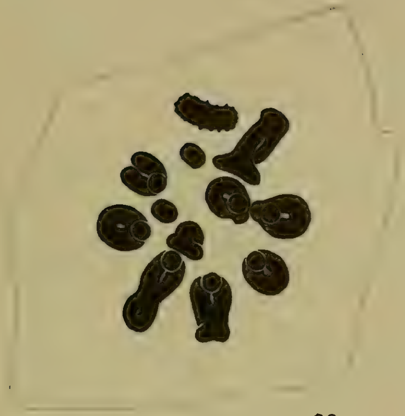

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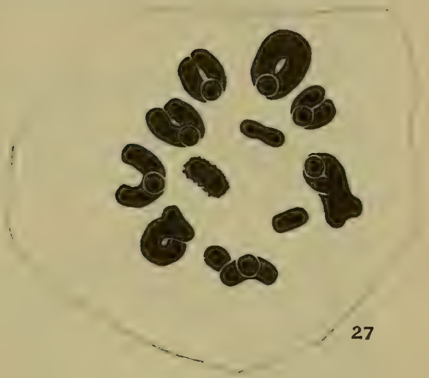
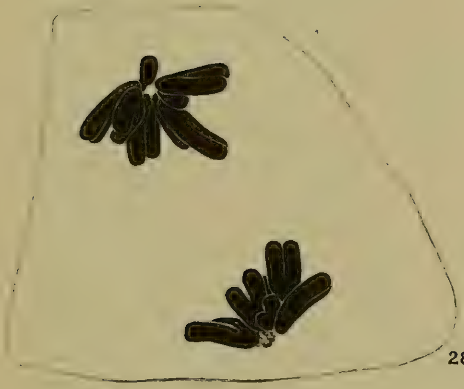
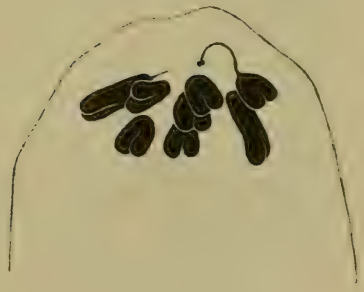


27



28



29

PLATE III

regards the spindle fibers as artifacts, produced in a homogeneous spindle substance by differential coagulation along hypothetical lines of force at the time of fixation. Neither does the observed behavior fit Bělăr's hypothesis (1929) that the chromosomal fibers are formed by a mucous secretion from the insertion points of the chromosomes which flows along underlying continuous fibers.

(4) The fourth feature is the actual presence of chromatin along the chromosome fiber (Figs. 22 and 23, tetrad at the left). The resemblance of these structures to satellites and constricted chromosomes is self-evident. Furthermore, the thicker basal parts of the chromosomal fibers often show the chromatic stain (hæmatoxylin, safranin and even Feulgen). In Fig. 16, for instance, the fibers on the tetrad at the right retain the safranin for nearly half their length. The same is true of the heavier portions of the fibers in all of the figures. As was stated earlier, all of the slides used with the single exception of one of *Dittopternis* (Fig. 29), were stained more than ten years ago and differentiated primarily for the chromosomes.

Passing finally to the anaphase, let us briefly consider the interzonal fibers.

Two or possibly three distinct structures are confused under this term in the literature. Mark (1881, p. 198) introduced the term in the following sentence: "Between the two zones of thickenings are stretched delicate nearly parallel threads, which I shall designate as *interzonal filaments*." The "two zones" are the anaphase groups of chromosomes, though the word chromosome had not yet been coined. Later (p. 230) he definitely states that the ". . . free ends (of the interzonal filaments) terminate in the lateral zones of spindle-fiber thickenings." Hermann (1891), on the contrary, concluded, from a study of mitosis in salamanders and other forms, that the connecting fibers apparent in anaphase are simply the fibers of the central spindle revealed by the separating chromatids. Hermann's view has been widely accepted by cytologists who failed to recognize the fact that in addition to, and morphologically quite distinct from, the fibers of the central spindle, there are connections frequently between the "free" ends of the chromatids as they separate in anaphase. These connections are thickest just as the ends begin to separate and at this time take the chromatin stain. As the separation increases the connection becomes more attenuated and, consequently, destains more rapidly so that in preparations differentiated for metaphase chromosomes all of the stain may be removed. A series of such stages is shown in Fig. 21 from first spermatocytes of *T. fallax*. The sixth tetrad from the left shows a connection which has persisted longer than usual. This tetrad is of further interest on account of the way in which the chromatin of the upper dyad is drawn out along the connecting fiber, a behavior directly comparable to the extension of chromatin along the chromosomal processes illustrated in Fig. 22. Ordinarily, in the Acrididæ, the interzonal connections separate (I do not believe that they break) near the mid-region at about the stage shown in the third tetrad from the left and are retracted each end into its own chromatid. To me, the action seems to be exactly what one would expect under the existing conditions; namely, flexible, viscous, protoplasmic rods are separating in such a manner that the final contact is end to end. As these ends are separating, it is readily conceivable that viscous protoplasm belonging to each might be drawn out between them to a varying degree, depending upon such factors as the rapidity of the movement and the viscosity of the chromatids. Whether or not interzonal fibers occur in normal, actively dividing cells may be questionable, but they certainly do occur in living cells which are sufficiently normal to complete division as well as in fixed preparations. If my conception is correct, they may well be lacking both in entire groups of organisms and in special cell divisions where the rigidity of the metaphase chromatids is adequate to prevent adhesion, or where the chromatids are separated widely. For instance, in the second maturation division of the short-horned grasshoppers, interzonal fibers are lacking entirely. This condition I believe to be due to the relatively wide separation of the chromatids when they come into the plate for this division.

In my material I find no evidence of a chromosomal sheath or of tubular connections between separating chromatids in anaphase such as Schrader describes in certain Hemiptera. In *Leptocoris* first spermatocyte anaphases fixed in Flemming's strong solution, I have found no interzonal fibers but a pathway such as the dense daughter chromosomes might leave when separating from each other in a fairly viscous cytoplasm if the clearer, more fluid part of the cytoplasm flows in between them as they move apart. This condition is essentially the same as Ellenhorn (1933, p. 300) reported in living cells from *Tradescantia* anthers, where the cytoplasmic viscosity had been increased by pressure sufficient to cause a reversible gelation which did not stop the mitotic process. Schrader (1934, p. 520) states that Ellenhorn, like himself, interprets the interzonal connections as tubes. Fundamentally, their conceptions are entirely different since Ellenhorn considers the internal connections to be transient canals through viscous cytoplasm and Schrader (1932, p. 537) believes them to be a chromophobic outer layer of the chromosome which is pulled out in the form of a tube between the separating daughter chromosomes and which may persist with the lumen obliterated until the formation of the spindle for the succeeding division.

A further phenomenon which must be concerned with the structure of the spindle, although I do not quite see its mechanism, should be considered here and that is the variation in size of the spindle which may be produced experimentally. Figures 24 to 28 are from first spermatocytes of *T. citrina.* Figures 24, 26, and 28 are from the same insect which was killed with cyanide fumes. Figures 25 and 27 are from another individual which was killed with xylol applied to the spiracles. The differences illustrated are characteristic. Cyanide gives short spindles with the centrioles well removed from the cell membrane and much of the cytoplasm apparently not involved, while xylol gives approximately twice as much distance between the centrioles which are consequently near the cell membrane with the spindle occupying nearly the entire cell. Correspondingly, the chromosomes are more crowded together in a smaller equatorial plate in the former case, as may be seen by comparing Figs. 26 and 27 from the same two insects. Even at the completion of the anaphase the distance between the poles in cyanide-killed animals is only slightly more than two-thirds of what it is in metaphase for an animal killed with xylol (Fig. 28).[7] Some question has existed as to which is the normal condition but recently an individual of *Dittopternis* killed with xylol gave both types of spindles. The short ones occur in a limited region which was injured, evidently, when the testis was removed. The cells have coarsely granular, muddy staining cytoplasm and in extreme cases the chromosomes had fused into a pycnotic mass characteristic of dying cells. Clearly, then the large spindles are the normal ones.

When one considers that less than five minutes previous to fixation both animals, from which the figures were taken, were presumably in the same condition so far as their mitotic spindles were concerned,[8] one marvels at a mechanism which permits such rapid rearrangement. If the chromosomal fibers are processes from the chromosomes, their rapid retraction is understandable. As to the astral rays and continuous spindle fibers, one can imagine elongate molecules to be involved. A. R. Moore (1935) has produced evidence for the existence of such elongate prestructural elements, molecules or micelles, in protoplasm by showing that the plasmodia of *Physarum* will pass through moist walls of hard filter paper with pores about 1 micron in diameter uninjured while they are killed if forced through silk gauze with pores less than 200 micra in diameter.

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[7] McClung (1918*a*) reported the relation of this condition to the method of killing the animal but illustrations were not given.

[8] This presumption is based on some fifty specimens killed with cyanide fumes and thousands with xylol.

Discussion

The observations of the earliest investigators in any given field frequently are strictly accurate and amazingly detailed. Probably because they alone approach the subject with a completely open mind.

It is interesting to note that Flemming (1882, Pl. VI, Fig. 3*b*) shows a late anaphase of a living "Leydischen schleimzelle" of a salamander larva with achromatic threads, which he states are clearly visible. The general accuracy of Flemming's observations is attested by his figures of mitosis from fixed material which far excel those of many later cytologists. Certainly, then, his statement that he saw achromatic threads, which his figure shows to be in the interzonal region, is to be taken at face value. Nevertheless, they might have been any one of three structures: (1) continuous spindle fibers, although this is not probable, (2) interzonal fibers which as drawn out parts of the chromosomes should be visible in living cells, since the chromosomes themselves are visible; (interzonal fibers, however, seldom persist until late anaphase); (3) mitochondria which are present in abundance at this stage in most forms and are readily visible in living cells.

Bělăr (1929, Pl. I, Figs. 14 and 15) shows photomicrographs of what is probably a similar condition in living first spermatocyte anaphases of *Chorthippus lineatus*. He interprets the apparent fibers as streaming mitochondria. On the other hand, the excellent ultraviolet photomicrographs of a similar stage in second spermatocytes of another grasshopper, *Melanoplus femur-rubrum*, by Lucas and Stark (1931), show nothing comparable to fibers in the interzonal region. These photomicrographs have been cited by some investigators as evidence that the mitotic spindle is homogeneous. In reality, they are evidences only that any structures which are present are completely permeable to ultraviolet rays in this species. Mitochondria almost certainly were present. In addition, the only anaphase figures are of second spermatocytes (Plate III, Figs. 27, 28) where, as I have mentioned previously, interzonal fibers are lacking. Incidentally, it should be noted that the refractive index of living chromosomes varies greatly in different species of Acrididæ. Consequently, some are more favorable than others for the study of mitosis in living cells.

Knowledge of the various spindle components has been augmented greatly by the work of Cleveland and his collaborators on the flagellate parasites of the wood roaches. There is essential agreement in our conclusions. The chief point of difference is in regard to the chromosomal fibers which Cleveland et al. believe to be of dual origin; the intranuclear part derived from the chromosomes and the extranuclear portion from the astral rays. In the grasshoppers, I am convinced that they are entirely chromosomal in origin. While such a divergence would not be surprising in organisms so widely separated, phylogenetically, as the flagellate Protozoa and the grasshoppers, the possibility that the extranuclear chromosomal fibers in these flagellates are put forth from the "chromatin knobs" on the nuclear membrane would explain some of the difficulties encountered on the assumption of Cleveland and his coworkers. For example, describing the anaphase movement of the chromosomes, they state (p. 234) ". . . the extranuclear chromosomal fibers are greatly shortened, while the intranuclear chromosomal fibers are not greatly altered (Figs. 53, 56–58). The shortening of the extranuclear chromosomal fibers is difficult to understand. If, as all other observations indicate, they are astral rays that have been converted into chromosomal fibers by becoming fastened to the knobs on the nuclear membrane, why do they shorten while the unattached astral rays do not?" If the knobs send processes (fibers) through the nuclear membrane to the centrioles such fibers would not be expected to behave as do the astral rays, since their origin would be from the chromosomes. The paragraph just quoted continues: "The unattached astral rays, as a rule, are considerably longer than the longest attached ones (extranuclear chromosomal fibers). This is due to the increased length of the astral rays after the intra- and extranuclear chromosomal fibers are united and will account for the fact that the extranuclear fibers sometimes appear slightly larger than the astral rays, if the chromosomal fibers increase in thickness as the astral rays increase in length." This explanation of the fact that the chromosomal fibers are thicker than the astral rays seems rather far-fetched. If the latter originate from the centrioles and the former from the chromosomes, correspondence in thickness would not be expected.

As is well known, certain workers believe all spindle fibers to be artifacts. Schrader (1932, 1934) reviews the work of a number of such investigators in a very fair and sympathetic manner and gives an adequate bibliography. Schrader's answer to such views is a series of experiments on the mitotic figures of higher forms which show that the components of the half spindle may be bent, independently of each other, by centrifuging and that such metaphases will complete their division; thus disproving, so far as the chromosomal fibers are concerned, the contention that the fibers seen in fixed preparations are artifacts caused by the differential coagulation along lines of force of a homogeneous spindle substance.

If one grants for the sake of argument that lines of force could cause such coagulation artifacts to result from fixation, one would at least expect them to be continuous from pole to pole and of uniform diameter, whereas the outstanding characteristics of chromosomal fibers are that they taper from their points of emergence from the chromosomes towards the poles, and that they are not continuous from pole to pole. In short, a great many of the arguments put forward by those who question the actual existence of spindle components of all categories would never have been advanced if their proponents had had adequate training in the study of mitosis in well-fixed material before undertaking the more difficult study of mitosis in living cells.

Perhaps a brief statement of my conception of a chromosome is in order. In the first place it is an individualized mass of living substance (protoplasm). We speak of the protoplasm of an amœba as being specialized into ecto- and endoplasm and the evidence indicates that the protoplasm of a chromosome is specialized in much the same way. The cortical part must meet its environment adequately. During metaphase, anaphase and early telophase the immediate environment is the cytoplasm and the condensed chromosome exposes the least possible surface. These stages are concerned primarily with increase in cell number and not with the welfare of the chromosomes as individuals. In late telophase the chromosomes form the nuclear membrane (Wenrich, 1916, pp. 86–88). That the chromosomes are the determining factors in this process is further indicated by the fact that in the spermatogonial telophases the sex chromosome of the short-horned grasshopper forms an almost separate and relatively much more commodious "vesicle" for itself while the euchromosomes are rebuilding the nuclear membrane.[9] From the late telophase through the "resting period" and up to the end of the prophase the immediate environment of the chromosomes is the nucleoplasm. In this sheltered situation they expose a greatly increased amount of surface and probably reach the apex of their functional activity and individual well-being. Even this protected environment changes as tissue differentiation progresses and this may well be one of the factors which limit the number of cell divisions, and consequently, the size of a given organ. Support for such a view is found in the fact that in mature tissue cells which resume mitotic activity to repair an injury, the cytoplasm first dedifferentiates. In brief, a chromosome is a living entity clearly capable of growth, of reproducing itself by longitudinal fission, and of amœboid movement.

Finally we come to the crucial point. Can the position of the chromosomal fibers change without inversions or translocations of parts of the chromosomes; in other words, can new loci arise and suppress the older ones? If so, what is the mechanism involved? The association of the chromosomal fibers with a fixed region of the chromosome is one of the most constant features of chromosomal organization. Cytogeneticists, however, have long known that experimental breakage and fusions of chromosomes due to irradiation, abnormal temperatures and unknown intrinsic causes may result in shifts in the location of the chromosomal fiber region so that it does not invariably occupy the same position in a given chromosome; but in such cases, the region maintains its characteristics whatever its location.

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[9] McClung (1918*b*) noted the increased diffusion and presumably greater activity of the sex-chromosome during the spermatogonial interphases.

The first well-founded case of an association of the chromosomal fibers with a different region (chromomere in this instance) is that noted by Wenrich (1916) in *Phrynotettix*. His figure 65h–m illustrates six tetrads of his type C_1. Those shown at h–j are dividing equationally, those at k–m reductionally. In the former case the ends marked by the polar granules (Pinney, 1908), as shown by a comparison with the earlier stages, are as usual oriented towards the poles. In the latter case the large distal granule, characteristic of the free end of the larger homologue in the spermatogonial telophases, is oriented towards one pole and the erstwhile free end of its homologue is, of necessity, directed towards the other pole. In other words, the chromosomal fibers have shifted from the ends of these homologues marked by the polar granules to what in the spermatogonia were the distal or free ends, one of which is marked by a pronounced "distal granule." Wenrich found this change to have taken place in approximately fifty per cent of the 928 first spermatocytes recorded in the individual under consideration. His close study of the earlier stages of this tetrad renders it highly improbable that any inversion of parts of a chromosome where the regions are so well-marked could have taken place in half of the cells without detection by so competent an observer.[10]

Wenrich also notes (p. 84) the critical point, that the polar granules, located at the spindle fiber ends of the euchromosomes, and the large distal granules of tetrads "B" and "C," as well as the whole of the sex-chromosome become roughened by the metaphase of the first spermatocyte division (Plate IX, Figs. 99 and 100) and suggests that some common physical or chemical properties underlie this correspondence in behavior.

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[10] Schrader, F. (1936), in a paper which appeared after this manuscript was sent to press, concludes on the basis of structure and staining reaction that the "kinetochore" is quite distinct from an ordinary chromomere. I have no doubt that the degree of specialization varies in different organisms. However, I cannot agree with his suggestion that the polar granules of *Phrynotettix* (Pinney, 1908, Wenrich, 1916) are not "kinetochores" simply because tetrad "B" possesses a distal as well as a polar granule. In my opinion this is exactly the mechanism by which tetrad "C" shifts its spindle fiber locus.

One physical characteristic marks all of the above structures as well as sex-chromosomes, chromoplasts and precocious chromomeres in general; their rhythm of condensation and diffusion is different from that of the rest of the chromatin, with the result that at the metaphase they are already beginning to become diffuse. The X-chromosome of *Stauroderus scaleris* has reached a vesicular telophase condition at the first spermatocyte metaphase (Corey, 1933). Likewise, the X-chromosome of *Notonecta indica* goes into the first spermatocyte metaphase in a diffused condition and with multiple spindle fibers as was shown by Browne (1916, Pl. 6, Fig. 95). Carlson (1936, p. 132) gives a good description of the process of diffusion of the "megameric" chromosomes in the cryptosome stage in seven related genera of short-horned grasshoppers. If precocity of definite chromomeres is associated with the ability to begin putting forth processes at the metaphase, a change (mutation) in the rhythm of activity of certain chromomeres might result in some which are usually relatively quiescent at the metaphase, speeding up and sending out processes capable of becoming chromosomal fibers with a resultant struggle for supremacy such as is exhibited by the X-chromosomes with multiple chromosomal fibers illustrated in Figs. 1 to 5. In my opinion, a specialized chromomere of each chromatid normally sends out a fine pseudopodial-like process at the metaphase which becomes the chromosomal fiber, but a change of rate of condensation and diffusion may result in a shifting of this function to a more precocious chromomere.

SUMMARY

The evidence presented is believed to justify the following conclusions: (1) Chromosomal fibers (half-spindle fibers) are in reality pseudopodial-like processes. They are put forth by definite chromomeres, therefore normally are fixed in position. (2) Due to mutation, using this term in a broad sense, some other chromomere may assume this function with a resultant change in position of the spindle processes which does not involve either inversion or translocation of chromosomal segments. (3) At anaphase the processes, beginning with the heavier basal parts, are reincorporated into the sister chromatids as they move towards the poles. (4) The interzonal fibers behave as one would expect under the existing conditions; namely, flexible, viscous protoplasmic rods are separating in such a manner that the final contact is end to end. As these ends separate the viscous protoplasm belonging to each is drawn out between them in varying degree depending on such factors as the rapidity of the movement and the density of the chromatid. (5) Both chromosomal fibers and interzonal fibers are integral parts of the chromosomes and return to them during anaphase without loss of chromatin or linear derangement of their constituents. The work is based on six species belonging to the following four genera of Acrididæ; *Trimerotropis, Circotettix, Derotmema* and *Brachystola.* In addition, the paper contains a summary of what are believed to be well-established facts concerning the various components of the mitotic spindle.

BIBLIOGRAPHY

BĔLĂR, KARL, 1929. Beiträge zur Kausalanalyse der Mitose. II. Untersuchungen an den Spermatocyten von Chorthippus (Stenobothrus) lineatus Panz. *Arch. f. Entw.-mech.,* **118**: 359.

BOWEN, R. H., 1920. Studies on insect spermatogenesis. I. *Biol. Bull.,* **39**: 316.

BROWNE, ETHEL N., 1916. A comparative study of the chromosomes of six species of Notonecta. *Jour. Morph.,* **27**: 119.

CARLSON, J. G., 1936. The intergeneric homology of an atypical euchromosome in several closely related Acrididæ (order Orthoptera). *Jour. Morph.,* **59**: 123.

CAROTHERS, E. ELEANOR, 1917. The segregation and recombination of homologous chromosomes as found in two genera of Acrididæ (Orthoptera). *Jour. Morph.,* **28**: 445.

CLEVELAND, L. R., S. R. HALL, ELIZABETH P. SANDERS AND JANE COLLIER, 1934. The wood-feeding roach Cryptocercus, its protozoa, and the symbiosis between protozoa and roach. *Memoirs Am. Acad. Arts and Sci.,* **17**: 185.

CLEVELAND, L. R., 1935*a*. The centriole and its rôle in mitosis as seen in living cells. *Science,* **81**: 598.

CLEVELAND, L. R., 1935*b*. The centrioles of Pseudotrichonympha and their rôle in mitosis. *Biol. Bull.,* **69**: 46.

CONKLIN, E. G., 1902. Karyokinesis and cytokinesis in the maturation, fertilization and clevage of Crepidula and other Gastropoda. *Jour. Acad. Nat. Sci. Phila.,* **12**: .

COREY, H. IRENE, 1933. Chromosome studies in Stauroderus (an orthopteran). *Jour. Morph.,* **55**: 313.

ELLENHORN, JAKOB, 1933. Experimental—photographische Studien der lebenden Zelle. *Zeitschr. f. Zellf.,* **20**: 288.

FLEMMING, F., 1882. Zellsubstanz, Kern und Zellteilung. Vogel, Leipzig.

HENKING, H., 1890. Untersuchungen über die ersten Ewtwicklungsvorgänge in den Eiern der Insekten. II. Über Spermatogenese und deren Beziehung zur Eientwicklung bei Pyrrhocoris apterus L. *Zeitschr. f. wiss. Zoöl.,* **51**: 685.

HERMANN, F., 1891. Beitrag zur Lehre von der Entstehung der karyokinetischen Spindel. *Arch. Mikro. Anat.,* **37**: 569.

HUETTNER, A. F. AND M. RABINOWITZ, 1933. Demonstration of the central body in the living cell. *Science,* **78**: 367.

JOHNSON, H. H., 1931. Centrioles and other cytoplasmic components of the male germ cells of the Gryllidæ. *Zeitschr. wiss. Zool.,* **140**: 115.

LUCAS, F. F. AND MARY B. STARK, 1931. A study of living sperm cells of certain grasshoppers by means of the ultraviolet microscope. *Jour. Morph.,* **52**: 91.

LUCAS, MIRIAM S., 1932. The cytoplasmic phases of rejuvenescence and fission in Cyathodinium piriforme. II. A type of fission heretofore undescribed for ciliates. *Arch. f. Protist.*, **77**: 407.

McCLUNG, C. E., 1918*a*. Some considerations regarding microscopical technique. *Anat. Rec.*, **14**: 265.

McCLUNG, C. E., 1918*b*. Possible action of the sex-determining mechanism. *Proc. Nat. Acad. Sci.*, **4**: 160.

MARK, E. L., 1881. Maturation, fecundation and segmentation of Limax campestris. *Mus. Comp. Zool. Harvard*, **6**: 173.

MONTGOMERY, T. H., 1905. The spermatogenesis of Syrbula and Lycosa, etc. *Proc. Acad. Nat. Sci. Phila.*, **57**: 162.

MOORE, A. R., 1935. On the significance of cytoplasmic structure in plasmodium. *Jour. Cell. and Comp. Physiol.*, **7**: 113.

PINNEY, M. E., 1908. Organization of the chromosomes in Phrynotettix magnus. *Kan. Univ. Sci. Bull.*, **4**: 307.

SCHRADER, F., 1932. Recent hypotheses on the structure of spindles in the light of certain observations in Hemiptera. *Zeitschr. f. wiss. Zoöl.*, **142**: 520.

SCHRADER, F., 1934. On the reality of spindle fibers. *Biol. Bull.*, **67**: 519.

SCHRADER, F., 1936. The kinetochore or spindle fiber locus in Amphiuma tridactylus. *Biol. Bull.*, **70**: 484.

WENRICH, D. H., 1916. The spermatogenesis of Phrynotettix magnus, with special reference to synapsis and the individuality of the chromosomes. *Bull. Museum Comp. Zoöl.*, **60**: 57.

WILSON, E. B., 1901. A cytological study of artificial parthenogenesis in sea-urchin eggs. *Arch. f. Entw.-mech.*, **12**: 529.

SOME FRESH WATER PROTOZOA WITH BLUE CHROMATOPHORES

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Among the Protista blue is one of the rarest of the colors. The blue-green algæ may be grass-green or shades of brown or red. A culture of a marine *Spirulina* I maintained for two years was constantly violet. Such variations in the color of the Myxophyceæ are due to combinations of chlorophyll, phycocyanin, and phycoerythrin, and to the make-up of the gelatinous envelope at times.

Some Protozoa have a blue or violet color diffused through the cytoplasm as the ciliate, *Stentor cœreuleus* Ehrenberg. Other Protozoa may occasionally have a blue color due to specific pigments contained in chromatophores. A single fresh-water rhizopod, *Paulinella chromatophora* Lauterborn, fulfills this condition, but most of the blue Protozoa are Mastigophora. Various marine Dinoflagellida have blue or blue-green plastids, but according to Eddy (1930) none of the fresh-water armored species in the United States are so colored and I have been unable to find a record of such a species of *Gymnodinium,* the unarmored genus. The genera *Chroomonas* and *Cyanomonas* of the Cryptomonadida have blue chromatophores but Smith (1933) does not record the occurrence of either in this country; Pascher (1913), however, holds *Cryptoglena americana* Davis to be identical with *Cyanomonas americana* Oltmanns. West and Fritsch (1927) record *Chroomonas nordstetii* Hansgirg as reported once from England. From such scanty records of their occurrence it would be imagined that the blue Protista are very rare.

In the past several years I have collected some hundreds of water samples from various field locations. Many of these have been centrifuged to concentrate their microörganisms and examined before great temperature or other changes could occur. In such a sample taken from Newtown Creek at Collingswood, New Jersey, in 1932, I found a blue flagellate in abundance. Since then I have found *Paulinella chromatophora,* a new species of *Chroomonas,* and three other new flagellates with blue chromatophores. I have also noted a rather widespread field occurrence of some of these organisms. Table I shows those which have occurred at nine locations.

From this table a comparative idea of the rareness of occurrence of these blue Protozoa may be obtained. It should be stated, however, that *Cyanomonas americana* has never been seen by me from but two stations, and the same is true of *Cyanomastix morgani.* Species of *Chroomonas,* however, are relatively common; thus bi-weekly samples from a small pool in South Mountain Reservation, Essex County, New Jersey, rarely failed to show some of these flagellates, while in 76 plankton samples from the Muscle Shoals area in Tennessee, Mississippi and Alabama, 21 contained one or more species and in a few they constituted the most abundant protozoan organism. This is a much more frequent occurrence than available records indicate. Their small size and activity prevents ready identification, unless they are concentrated in some manner.

Protozoa with Blue Chromatophores Occurring at Nine Sampling Stations

Name of organism	Locality where found								
	Reservation Pool, Millburn, N. J.	Raritan River, N. J.	Pond, Woods Hole, Mass.	Reelfoot Lake, Tenn.	Reservoir, Wilson Dam, Ala.	Lake Wilson, Ala.	White's Lake, Pickwick Dam, Tenn.	Collier Slough, Florence, Ala.	Yellow Creek, Miss.
Paulinella chromatophora								×	×
Chroomonas pulex	×	×	×	×	×				×
Chroomonas nordstetii	×					×		×	
Chroomonas cyaneus	×				×	×	×	×	×
Cyanomonas americana									×
Cyanomonas cœreuleus							×		
Cyanomastix morgani									×
Gymnodinium limneticum				×		×			

In all these Protozoa the color is a bright blue, localized in chromatophores from which it diffuses out as a blue liquid after death of the organism. No experimental work on its nature has been done, but it is brighter than extracted phycocyanin.

Paulinella chromatophora Lauterborn. Plate I, Fig. 1.

This rhizopod was found at three stations in the spring of 1936, two of them shown in Table I. Its recurrence was several times noted at one of these. All the stations were clear streams with considerable debris and an abundance of filamentous blue-green algæ. Hydrogen ion concentrations were 6.2, 6.8, and 7.4. The animals conformed rather closely to the published descriptions, but are more pyriform in shape and with nine rows of plates instead of eleven or twelve. The cell does not fill the test and the cytoplasm is clear and homogenous. The nucleus is visible in the upper portion. All animals had two contractile vacuoles, definite in location as shown. The few pseudopodia are long and branch a few times. They are moved sluggishly. The two chromatophores are curved bands, decidedly blue with no pyrenoids. It is unlikely that they are symbiotic algæ as Kudo (1931) suggests, for every individual contained two; eighteen in one sample alone were examined.

Cyanomastix morgani, gen. nov., spec. nov. Plate I, Figs. 2, 3.

This organism occurred rather abundantly in a fresh water lime sink pond in Morgan County, Alabama, during September, 1935. It is difficult to classify, for its color places it in the Phytomastigoda, and its thick membrane, lack of a gullet and simple vacuole system should place it in the order Phytomonadida. While the peculiar shape and color of the chromatophores, and lack of a stigma are exceptional to the Chlamydomonadidæ, I believe it should be placed at least provisionally in that family. No resting stages or reproductive phases were observed.

Organisms practically uniform in size, 15 to 20 microns in length, 8 to 15 in width, slightly pyriform in shape. Membrane thick with five shallow grooves at round end, a small opening at top. Cytoplasm homogenous with an anterior nipple from which emerge two slightly subequal flagella, the longer about cell length. A contractile vacuole is just below this nipple. No nucleus was observed. All individuals had two chromatophores, band-shaped, twisted or turned, a vivid blue green. On them are a few large bodies resembling pyrenoids with irregular edges. The only noticeable cell inclusions are small spheres, probably oil.

Locomotion is rapid, rather like that of *Chlamydomonas.* The organisms also have the trick of attaching themselves by the anterior nipple, with the flagella opposed. Nutrition seemed to be holophytic.

Chroomonas setoniensis, spec. nov. Plate I, Figs. 4, 5.

This species conforms to the generic description as given by Pascher (1913). In size it is the largest member of its genus, attaining a length of 20 microns. Its flattened oval form, deeply insunk gullet and size seem to merit calling it a new species. It has been common at two stations in the Muscle Shoals area, and in the pool in South Mountain Reservation referred to above, it appeared constantly during several months in considerable numbers.

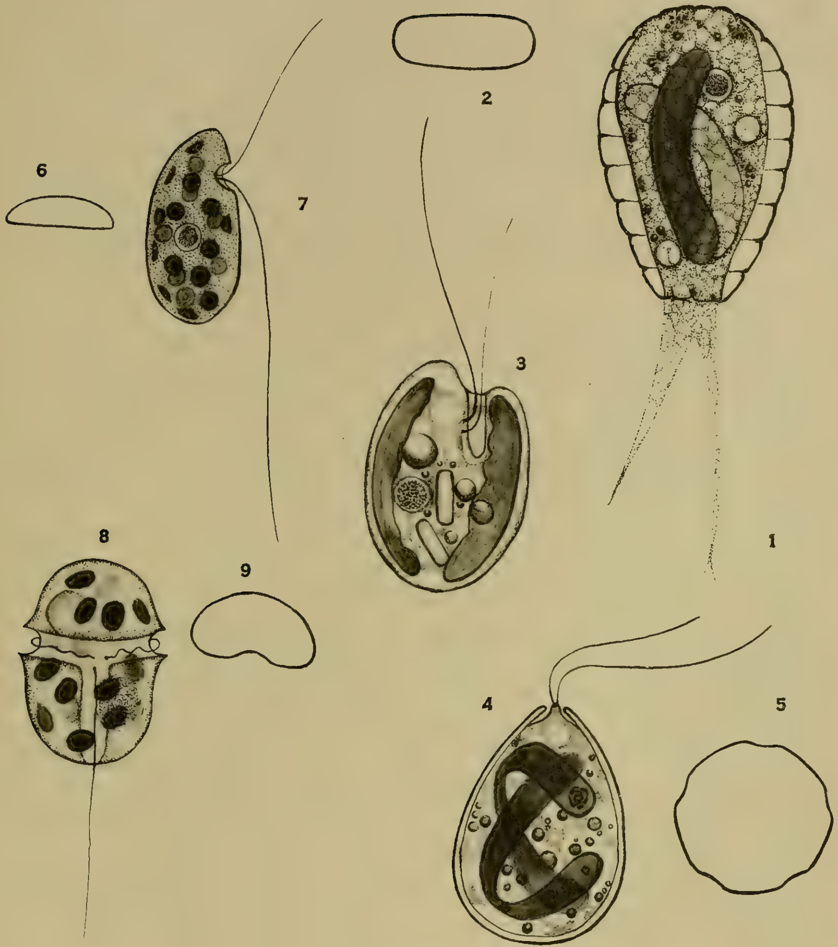

PLATE I

Cell constant in shape, flattened, oval, slightly obliquely truncate at the anterior end, with a deeply insunk gullet or mouth depression, open along one side. About 20 microns long, 15 wide and 8 thick. Two subequal flagella emerge from the mouth depression, the longest about 25 microns long, the shorter about 20. The single chromatophore is bright blue, having the form of a peripheral cylinder, split from anterior to posterior end, with irregular edges. One simple contractile vacuole near the point of emergence of the flagella. The nucleus is median with a thin membrane and no peripheral chromatin granules. Stained preparations show the flagella to end in small basal bodies, with no visible rhizoplast. There are several types of inclusions, spheres which might be oil, and bodies resembling paramylum. It swims rapidly revolving on its main axis. No evidence of reproduction has been seen.

Cyanomonas cœreulus, spec. nov. Plate I, Figs. 6, 7.

This organism has been seen in only two plankton samples; one from a lake, small and partly shaded, in Morgan County, Alabama, the other from a lake in Hardin County, Tennessee, at Pickwick Dam. This water was densely shaded by cypress and tupelo trees.

Cell somewhat egg-shaped, but flattened on one side. The anterior end is pointed. There is a small depression anteriorly, on the right side; two flagella emerge here. The longer is carried backward, about 20 to 25 microns long, and does not trail on the substratum at all times. The anterior flagellum is about cell length. The animal varies somewhat in size, 15 to 20 microns long and about half as wide. It is somewhat metabolic in that it assumes a discoid shape at times, as when changing direction of movement. The pellicle is not evident and its surface is smooth. The nucleus is small and central; there are no visible cell inclusions but the cytoplasm is finely homogeneously granular. No contractile vacuole is to be found. There are from 15 to 25 bright blue discoid chromatophores, without pyrenoids, small and peripherally located. Its nutrition and reproduction were not noted.

Davis (1894) described a blue-green motile cell from the salt marshes of the Charles River in Massachusetts as *Cyanomonas americana.* He thought it to be a motile cell of the blue-green alga *Polycystis pallida.* In general this organism is like his, except that the depression from which the flagella emerge is lateral instead of anterior, and there are no eyespots. A few flagellates more like his were found in a pool in Tishomingo County, Mississippi, in the summer of 1935, but could be studied only superficially. They came from a small pool which did not seem to have any blue-green algæ in it, and I have found nothing to connect these blue-green or blue flagellates with such algæ. On the contrary, they exhibit a high degree of organization.

Gymnodinium limneticum, spec. nov. Plate I, Figs. 8, 9.

This was the most common dinoflagellate at one station on Reelfoot Lake, Tennessee, in September, 1935. It was eaten in considerable numbers by the larvæ of malarial mosquitoes there.

Cell 25 to 35 microns long, somewhat dorsiventrally flattened, but a regular oval in outline. Hypocone somewhat larger than the epicone. Transverse furrow wide, deep, with no displacement. The longitudinal furrow does not enter the epicone. Trailing flagellum about one and a half times the body length. Commonly a large pusule in the hypocone, the nucleus being subcentral. The blue color is due to 8 to 12 oval discoid chromatophores scattered through the cell. No oil or other inclusions were noted, and as no food bodies were found it is inferred that nutrition is holophytic. The animal is naked and the surface is smooth. No reproduction was seen.

These blue flagellates present a perplexing question as to the significance of their color and its development. It appears improbable that they have any close relationship to the blue-green algæ; they are too highly organized. Morphologically their relationship to amœboid and flagellate Protozoa is unmistakable. Since all appear to nourish themselves holophytically, it is perhaps simplest to regard them as species in which chromatophores have developed containing a blue pigment in place of, or in addition to, chlorophyll.

REFERENCES

Davis, Bradley M., 1894. Notes on the life history of a blue-green motile cell. *Bot. Gaz.,* **19**: 3.

Eddy, Samuel, 1930. The fresh-water armored or thecate dinoflagellates. *Trans. Am. Micros. Soc.,* **49**: 277.

Kudo, R., 1931. Handbook of Protozoölogy. C. C. Thomas. Springfield, Illinois.

Lackey, J. B., 1932. Oxygen deficiency and sewage Protozoa, with descriptions of some new species. *Biol. Bull.,* **63**: 287.

Pascher, A., 1913. Die Susswasserflora Deutschlands, Osterreich, und der Schweiz. 2. Flagellatæ. 2. Gustav Fischer, Jena.

Smith, Gilbert M., 1933. The Fresh-water Algæ of the United States. McGraw Hill Book Co. New York.

West, G. S., and F. E. Fritsch, 1927. A Treatise of the British Fresh-water Algæ. Cambridge University Press, London.

THE MOLECULAR WEIGHTS AND PH–STABILITY REGIONS OF THE HEMOCYANINS

INGA–BRITTA ERIKSSON–QUENSEL AND THE SVEDBERG

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Previous ultracentrifugal investigations carried out in this laboratory have brought to light a number of different molecular weights and sedimentation constants among the hemocyanins (Svedberg and Chirnoaga, 1928; Svedberg and Heyroth, 1929, *a*, *b;* Svedberg and Eriksson, 1932; Svedberg, 1933; Svedberg and Hedenius, 1933; Svedberg and Hedenius, 1934). It has also been observed that a change in the pH of the solution often causes the appearance of one or several new molecular species and the disappearance—partial or complete—of the species present before the change was brought about (Svedberg and Hedenius, 1934; Svedberg, 1934, *a*, *b*, *c*, *d*). The pH-stability regions characterized in this way have often been found to be quite different even in the case of hemocyanins possessing the same molecular weight at the isoelectric point. This circumstance seems to be of considerable interest from a biological point of view and we have therefore made an attempt to study in some detail the molecular weights and pH-stability regions of a number of hemocyanins in order to find out whether the diagram giving the molecular weight (or the sedimentation constant) as a function of the pH of the solution could be used to define an animal genus or species.

METHOD

The ultracentrifugal technique developed for the determination of molecular weights and sedimentation constants has been described elsewhere (Svedberg, 1934, *a*, *b*, *c*, *d*). In the case of sedimentation equilibrium measurements the molecular weight is given by the formula:

$$M = \frac{2RT \ln c_2/c_1}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)} \tag{1}$$

where M = molecular weight, R = gas constant, T = absolute temperature, c_2 and c_1 = concentrations of solute, V = partial specific volume, ρ = density of solvent, x_2 and x_1 = distances to the centre of rotation, ω = angular velocity.

For the determination of c_2/c_1 a procedure based upon measurements of light absorption has previously been used almost exclusively. A new method for determining the concentration gradient utilizing the change in refractive index has been developed by O. Lamm (1933) and has in most cases been found to be superior to the absorption method in its present shape. The refraction method in its two forms (the scale-method and the slit-method) has therefore been used in this investigation.

Sedimentation equilibrium measurements require the expenditure of very much time and work and such determinations can therefore hardly be used for mapping out completely the pH-stability regions. In cases where more than one molecular species is present in the solution the equilibrium method has also the great drawback that it is unable to give definite information about the number and nature of these components. We have therefore used the sedimentation velocity method for collecting the data necessary for tracing the pH-stability pictures supplementing these measurements with equilibrium determinations. In the following diagrams the changes in the sedimentation constant will accordingly indicate the dissociation reactions occurring at different pH values. The sedimentation constant results from a measurement of sedimentation velocity by reducing the directly observed values to standard conditions with regard to centrifugal force, buoyancy and viscosity:

$$s = dx/dt \; 1/\omega^2 x \; \eta/\eta_0 \frac{(1 - V\rho_0)}{(1 - V\rho)} \qquad (2)$$

where dx/dt = observed sedimentation velocity, ω = angular velocity, x = distance from centre of rotation, η = viscosity of solvent, η_0 = viscosity of water at 20° C., V = partial specific volume of solute, ρ = density of solvent, ρ_0 = density of water at 20° C.

In order to minimize the danger of causing artificial changes in the state of aggregation all the hemocyanins have been studied as they occur in the blood without any treatment other than dilution with NaCl or buffer solutions. In the case of the crustaceans the fibrin has been removed by shaking the blood with glass beads. As a rule the light absorption method has been used in the determination of the sedimentation constants (λ = 366 mμ, nickel oxid glass filter or the region λ = 250–290 mμ, chlorine and bromine filter) but some check runs with the refraction method have been carried out. With regard to the details of procedure the previous communication (Svedberg and Hedenius, 1934) should be consulted.

Recent work in this laboratory has shown that some of the earlier sedimentation equilibrium measurements have given too low molecular weight values (probable causes of error: systematic deviations in the evaluation of the photographic blackenings, incomplete attainment of equilibrium). The discrepancy believed to exist between the frictional constants deduced from sedimentation velocity and sedimentation equilibrium measurements on the one hand and diffusion measurements on the other (Tiselius and Gross, 1934; O. Lamm and A. Polson, 1936) therefore vanishes and the possibility of computing molecular weights by combining sedimentation velocity and diffusion measurements as pointed out long ago by one of us (Svedberg, 1925) and used for such computations at the earlier stages of our investigations (e.g. Svedberg, 1927) is accordingly actualized.

The molecular weight is given by:

$$M = \frac{RTs}{D(1 - V\rho)} \tag{3}$$

where D = diffusion constant and the other symbols have their previous meaning.

A systematic study of the diffusion constants of the proteins undertaken by A. Polson has furnished a number of data also for the hemocyanin. His diffusion constants combined with our sedimentation constants according to equation (3) give molecular weight values which agree very well with those calculated from our sedimentation equilibrium measurements (see Table XXVI).

SEDIMENTATION CONSTANTS[1]

The main part of the work consisted in the carrying out of sedimentation velocity measurements at different pH-values. From these data the pH-stability curves were traced. In some cases the reversibility of the dissociation reactions have been tested.

Arthropoda

Hemocyanin is found in the blood of the crustaceans and the Arachnomorpha, but not in that of the myriapods and the insects. We have only studied representatives of the subclasses Malacostraca and Xiphosura, the species belonging to the other subclasses yielding too little blood for an extended investigation.

Crustacea: Malacostraca

The respiratory proteins contained in the blood of the following animals belonging to the order Malacostraca of the class Crustacea have been investigated: *Pandalus borealis*, *Palinurus vulgaris*, *Nephrops norvegicus*, *Homarus vulgaris*, *Astacus fluviatilis*, *Cancer pagurus*, *Carcinus mænas*. The two sedimentation constants 16 and 23 are characteristic of the group. In *Pandalus* and *Palinurus* the sedimentation constants for the main components are 16.4 and 17.4. In all the others the "normal" sedimentation constant is about 23 and dissociation products with sedimentation constants around 16 are formed. *Pandalus* shows an aggregation product with the sedimentation constant 22.9. All the final alkaline-splitting products have sedimentation constants approximating 5.

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[1] The sedimentation constants are expressed in units of 10^{-13}. Centrifugal force is expressed in terms of the gravitational constant.

The letters A–K are used to characterize the different hemocyanin components. Components having the same sedimentation constant are designated by the same letter.

A + in the column of a table indicates that the component in question has been observed but that it was impossible to calculate its sedimentation constant.

TABLE I

Pandalus borealis

Dilution of blood, 15 times; centrifugal force, 200,000 (speed 52,000 r.p.m.); thickness of column of solution, 0.6 cm.; source of light, mercury arc; light filter, chlorine and bromine; plates, Imperial Process; exposure time, 30 seconds; aperture of lens, F : 36; developer, metol-hydroquinone, 1 minute.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	
HAc, NaAc, NaCl	3.6	0.22		16.1	Containing inhomogeneous components with $s_{20} = 12.0$ and 4.7
" " "	4.0	0.22		18.4	Containing inhomogeneous components with $s_{20} = 4.7$
" " "	4.2	0.22	22.3	16.9	
" " "	4.6	0.22	22.4		Inhomogeneous
" " "	5.0	0.22	21.9		Inhomogeneous
" " "	5.2	0.22		17.6	Slightly inhomogeneous
" " "	5.5	0.22		17.4	
KH_2PO_4, Na_2HPO_4, NaCl	6.0	0.22		18.4	
" " "	6.8	0.22		17.4	
" " "	6.8	0.22		17.0	
" " "	6.8	0.22		17.7	
" " "	7.4	0.22		16.4	
" " "	8.0	0.22		16.9	
KH_2PO_4, $Na_2B_4O_7$, NaCl	8.5	0.27		17.0	
" " "	9.0	0.27		17.9	
Na_2CO_3, $Na_2B_4O_7$, NaCl	9.5	0.25		17.7	
" " "	9.5	0.25		17.4	
" " "	10.0	0.25		17.0	
" " "	10.5	0.25		16.6	7 times diluted; NiO-filter
" " "	10.8			18.1	Inhomogeneous lower part
			22.9	17.4	

FIG. 2*a*.

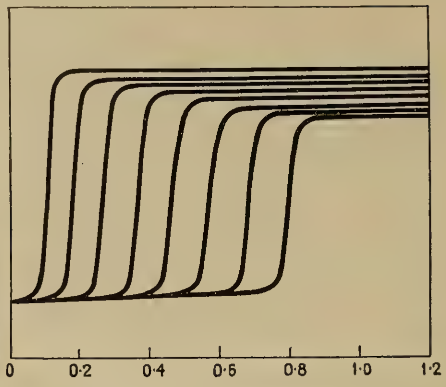


FIG. 2*b*.

FIG. 2. Sedimentation pictures (*a*) with photometric records (*b*) for hemocyanin from *Palinurus vulgaris* at pH 9.0 (s_{20} = 16.4); centrifugal force 120,000; time between exposures 5 minutes.

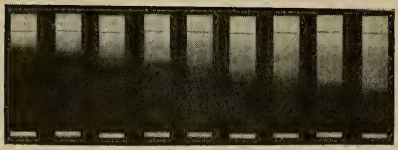


FIG. 3*a*.

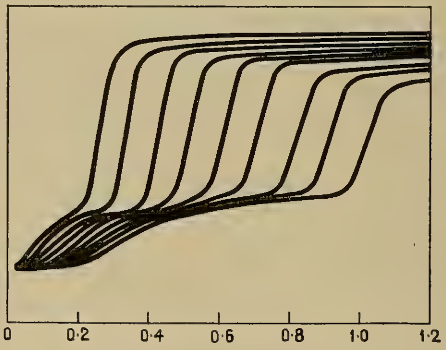


FIG. 3*b*.

FIG. 3. Sedimentation pictures (*a*) with photometric records (*b*) for hemocyanin from *Palinurus vulgaris* at pH 10 (s_{20} = 16.4 and 4.10); centrifugal force 280,000; time between exposures 5 minutes.

Pandalus borealis

Kristineberg, Sweden

The main component of this hemocyanin, component H, has the sedimentation constant 17.4. It is present in the blood from pH 3.6–10.8 with the exception of the pH region 4.2–5.0. It is homogeneous with regard to molecular weight from pH 5.5–10.5. At 10.8, 4.0, and 3.6 a large part of H dissociates into smaller molecules. In the region 4.2–5.0 the blood contains a higher very inhomogeneous component, G, the sedimentation constant of which is 22.9. Figure 1 (Plate I) gives the pH-stability diagram.

Palinurus vulgaris

Roscoff, France

In the pH-region 3.6–9.4 one molecular species is found in the hemocyanin (component H). This has the sedimentation constant 16.4 and is perfectly homogeneous. At pH 9.4 it dissociates to some extent into particles with the sedimentation constant 4.10 (comp. K). The low-molecular fraction increases with pH. At 10.8 H has disappeared. K is rather inhomogeneous and becomes more so in the more alkaline solutions. Figures 2 and 3 give examples of sedimentation runs and Fig. 4 (Plate I) the pH-stability diagram.

TABLE II

Palinurus vulgaris

Dilution of blood 5 times; centrifugal force, 150,000 (speed 45,000 r.p.m.); in the last two runs 250,000 (speed 59,000 r.p.m.); thickness of column of solution, 2.2–1.0 cm.; light filter, nickel oxid glass; time of exposure 10–15 seconds; other conditions as in Table I.

Solvent	pH of solvent	Total molar	s_{20} H	s_{20} K	
NaAc, HAc, NaCl	3.6	0.20	17.3		
" " "	4.0	0.20	17.4		
" " "	4.6	0.20	16.6		
" " "	5.0	0.20	16.7		
" " "	5.5	0.20	16.2		
Na_2HPO_4, KH_2PO_4, NaCl	6.0	0.20	16.8		
" " "	6.8	0.20	15.6		
" " "	6.8	0.20	16.6		
" " "	7.4	0.20	16.3		
" " "	8.0	0.20	15.6		
$Na_2B_4O_7$, KH_2PO_4, NaCl	8.5	0.20	15.7		
" " "	9.0	0.20	15.7		
" " "	9.4	0.20	16.8	+	
" Na_2CO_3, NaCl	9.7	0.20	17.3	5.40	
" " "	10.0	0.20	16.9	5.61	
" " "	10.1	0.20	17.1	3.89	
" " "	10.1	0.20	15.2	5.68	
" " "	10.3	0.20	16.7	5.81	
" " "	10.6	0.20	15.7	5.32	
Na_2HPO_4, NaOH, NaCl	10.8	0.30		5.47	Inhomogeneous
" " "	11.3			3.83*	Inhomogeneous
			16.4	4.10	

* Not used for the calculation of mean value.

Nephrops norvegicus

Kristineberg, Sweden

The main component G of the hemocyanin is present alone in the blood in the pH-region 4.0–8.0. It is homogeneous with regard to molecular weight and has the sedimentation constant 24.5. At pH 8.0 G is partly dissociated into component H with the sedimentation constant 17.1. Dissociation goes further at pH 10.2 and component K with sedimentation constant 5.97 is formed. Thus in the pH-region 8.0–10.2 the hemocyanin consists of the components G and H, in the pH-region 10.2–11.4 of G, H, and K. The decreasing of the sedimentation constant of G in the latter region is probably due to a change in shape of the molecule. At 11.4 G and H are both completely dis-

TABLE III

Nephrops norvegicus

Dilution of blood, 10 times; centrifugal force, 150,000 (speed 45,000 r.p.m.); in the last run 220,000 (speed 56,000 r.p.m.); other conditions as in Table I.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	s_{20} K	
HAc, NaAc, NaCl......	3.6	0.22		17.6		Very inhomogeneous
" " "	4.0	0.22	23.9			Slightly inhomogeneous
" " "	4.5	0.22	24.0			
" " "	5.0	0.22	24.6			
" " "	5.5	0.22	23.4			
KH_2PO_4, Na_2HPO_4, NaCl	6.0	0.22	25.8			
" " "	6.5	0.22	25.1			
" " "	6.8	0.22	23.9			NiO-filter
" " "	6.8	0.22	25.5			
" " "	7.4	0.22	25.1			
" " "	7.7	0.22	24.3			
" " "	8.0	0.22	25.1	+		
KH_2PO_4, $Na_2B_4O_7$, NaCl.	8.5	0.27	23.5	+		
Na_2CO_3, $Na_2B_4O_7$, NaCl.	9.5	0.25	24.1	16.7		
" " " .	10.0	0.25	24.3	+		
" " " .	10.2	0.25	23.6*	18.6	5.98	
" " " .	10.5	0.25	22.5*	17.7	6.46	
" " " .	10.7	0.25	19.1*	16.6	5.92	
Na_2HPO_4, NaOH, NaCl.	11.0	0.30	19.9*	15.8	5.42	
" " " .	11.4	0.28			6.08	Containing inhomogeneous component with $s_{20} = 3.54$
" " " .	11.7	0.28			8.95	Very inhomogeneous
			24.5	17.1	5.97	

* Not used for calculation of the mean value.

sociated and a still more low-molecular product appears with the sedimentation constant 3.5. At pH 11.7 the material is completely inhomogeneous. Figure 5 (Plate I) gives the pH-stability diagram.

TABLE IV

Homarus vulgaris

Dilution of blood 6–10 times; centrifugal force 120,000–220,000 (speed 40,000–55,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	s_{20} K	
Undiluted blood			13.5*			High viscosity
Diluted 3 times 0.1-mKCl			21.0*			High viscosity
HCl, Na-citrate, NaCl	3.4		23.3			
HAc, NaAc, KCl	4.0	0.12	24.5			Purified hemocyanin (Am_2SO_4-prec.)
HAc, NaAc, NaCl	4.0	0.20	22.8			
HAc, NaAc, KCl	5.0	0.12	22.6			
HAc, NaAc, NaCl	5.0	0.20	23.4			
KH_2PO_4, Na_2HPO_4, NaCl	6.0	0.20	22.5			
" " "	6.8	0.20	23.6			
KH_2PO_4, Na_2HPO_4, KCl	6.8	0.12	20.7			
" " "	8.0	0.12	22.6			
KH_2PO_4, $Na_2B_4O_7$, NaCl	9.0	0.20	21.9			
NaOH, Na_2HPO_4, KCl	9.8	0.15	23.4	16.9		
$Na_2B_4O_7$, Na_2CO_3, NaCl	10.0	0.25		17.3		
" " "	10.1	0.23		14.8		
" " "	10.0	0.20	22.2			
NaOH, Na_2HPO_4, NaCl	10.2	0.05	19.3	15.5		
$Na_2B_4O_7$, Na_2CO_3, NaCl	10.3	0.20		15.5		
" " "	10.3	0.20	21.8			
" " "	10.6	0.17		16.0	+	
NaOH, Na_2HPO_4, NaCl	10.7	0.06		15.6	6.64	
$Na_2B_4O_7$, Na_2CO_3, NaCl	10.5	0.20	21.5*			
" " "	10.3	0.20	19.7*			
" " "	10.7	0.20	19.8*	16.8		
Na_2CO_3, $Na_2B_4O_7$, NaCl	10.8	0.20		16.6		
NaOH, Na_2HPO_4, NaCl	11.0	0.06		13.8*	+	
" " "	11.0	0.32		12.2*	9.72	Inhomogeneous
" " "	11.4	0.07			5.81	Inhomogeneous
			22.6	16.1		

* Not used for calculation of mean value.

Homarus vulgaris

Havstensund, Sweden

The sedimentation constant of the main component G is 22.8. G is present alone in the blood from pH 3.4 to 9.8. At pH 9.8 and above a dissociation product, H, is formed with the sedimentation constant 16.1. When both G and H are present together it is difficult to determine their separate sedimentation constants. Generally G is observed in solutions of pH up to around 10.5 and H up to 11. At pH 11.4 the sedimentation picture shows only low-molecular inhomogeneous material. Figure 6 (Plate I) gives the pH-stability diagram.

Astacus fluviatilis

Upland, Sweden

The hemocyanin shows more than one component in the whole pH-range investigated. In the pH region 3.6–10, 80–85 per cent of the

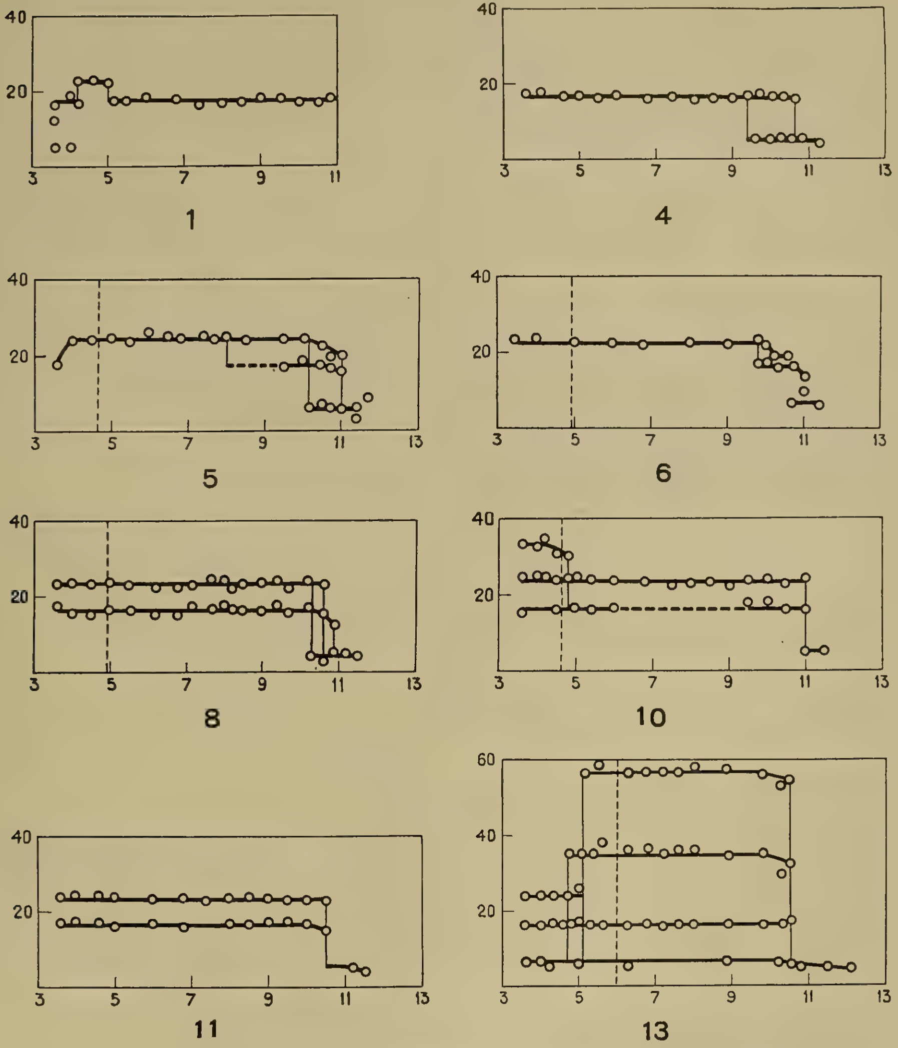
TABLE V

Astacus fluviatilis

Dilution of blood 10 times; centrifugal force 150,000 (speed 45,000 r.p.m.); for the last three runs 250,000 (speed 59,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	s_{20} K	
HAc, NaAc, NaCl.............	3.6	0.20	23.2	17.5		Inhomogeneous
" " "	4.0	0.20	23.4	15.4		
" " "	4.6	0.20	23.1	15.1		
" " "	5.0	0.20	24.2	16.7		
KH_2PO_4, Na_2HPO_4, NaCl.......	5.6	0.20	23.1	16.4		
" " "	6.2	0.20	22.3	15.7		
" " "	6.8	0.20	22.3	14.8		
" " "	7.2	0.20	23.1	16.7		
" " "	7.7	0.20	24.5	16.6		
" " "	8.0	0.20	23.7	16.7		
KH_2PO_4, $Na_2B_4O_7$, NaCl........	8.2	0.25	21.8	16.1		
" " "	8.5	0.25	23.6	16.3		
" " "	9.0	0.25	23.7	16.1		
Na_2CO_3, $Na_2B_4O_7$, NaCl........	9.4	0.25	24.0	17.5		
" " "	9.7	0.25	22.1	15.4		
" " "	10.2	0.25	24.1	16.6		
" " "	10.3	0.23	24.4	17.3	3.73	
" " "	10.6	0.21	22.9	15.6	2.37	
NaOH, Na_2HPO_4, NaCl........	10.9	0.27		12.5*	5.15	
" " "	11.2	0.27			4.65	Inhomogeneous
" " "	11.5	0.27			4.19	"
			23.3	16.3	4.02	

* Not used for calculation of mean value.



1 4 5 6 8 10 11 13

PLATE I

pH-stability diagrams for hemocyanins of species listed below. Abscissæ in all figures, pH; ordinates, s_{20}. The dotted lines in Figs. 5, 6, 8, 10 and 13 indicate the positions of the isoelectric points.

FIG. 1. *Pandalus borealis.*
FIG. 4. *Palinurus vulgaris.*
FIG. 5. *Nephrops norvegicus.*
FIG. 6. *Homarus vulgaris.*
FIG. 8. *Astacus fluviatilis.*
FIG. 10. *Cancer pagurus.*
FIG. 11. *Carcinus mœnas.*
FIG. 13. *Limulus polyphemus.*

protein has the sedimentation constant 23.3 (component G). The other component present, H, has the sedimentation constant 16.3. Around pH 10 the amount of H starts to increase, G to decrease. At

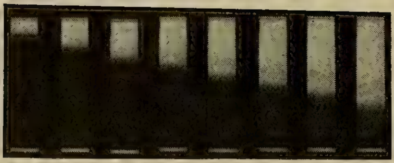


FIG. 7*a*.

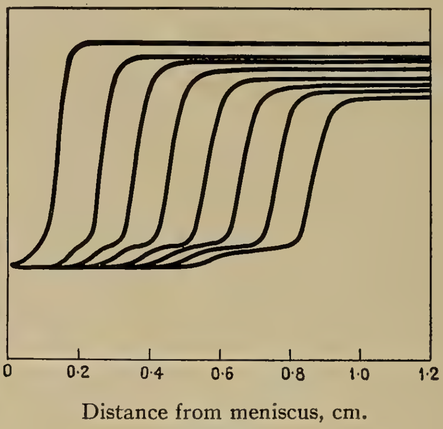


FIG. 7*b*.

FIG. 7. Sedimentation pictures (*a*) with photometric records (*b*) for hemocyanin from *Astacus fluviatilis* at pH 7.7 (s_{20} = 23.3 and 16.3); centrifugal force 150,000; time between exposures 5 minutes.

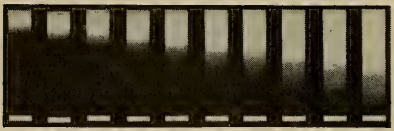


FIG. 9*a*.

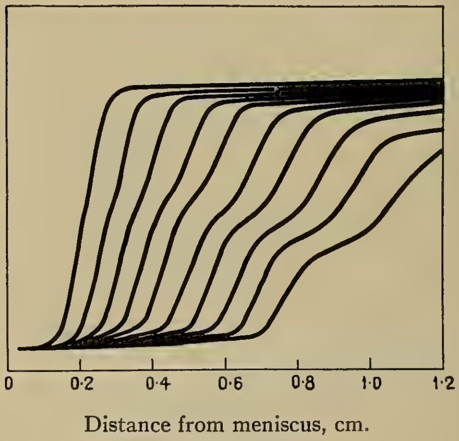


FIG. 9*b*.

FIG. 9. Sedimentation pictures (*a*) with photometric records (*b*) for the hemocyanin from *Cancer pagurus* at pH 4.2 (s_{20} = 32.7, 23.6, and 16.4); centrifugal force 120,000; time between exposures 5 minutes.

pH 10.3 a low molecular component, K, is formed. In a narrow pH-range, 10.3–10.6 G, H, and K appear together. At pH 10.9 G has totally disappeared and there is very little left of H. In more alkaline buffers all of the material is low-molecular and inhomogeneous. Figure 7 gives an example of a sedimentation run and Fig. 8 (Plate I) the pH-stability diagram.

Cancer pagurus

Havstensund, Sweden

The main component, G, of this blood has the sedimentation constant 23.6 and is observed in the wide pH-range 3.6–11.0. G is always accompanied by another component H with the sedimentation constant 16.4. H is present only in very small quantity. In the pH range 3.6–4.8 appears a third component, F, aggregation product of G, with the sedimentation constant 32.7. At pH 11.0 component K with sedimentation constant 4.77 is observed together with G and H. At

TABLE VI

Cancer pagurus

Dilution of blood 6 times; centrifugal field 115,000 (speed 40,000 r.p.m.); in the last run 220,000 (55,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} F	s_{20} G	s_{20} H	s_{20} K	
HCl Na-citrate, NaCl....	3.0	0.20					Aggregated and inhomogeneous
HAc, NaAc, NaCl.......	3.6	0.20	32.7	24.7	15.3		
" " "	4.0	0.20	32.5	24.9	+		
" " "	4.2	0.20	34.6	25.0	+		
" " "	4.5	0.20	31.0	24.2	16.0		
" " "	4.8	0.20	29.9*	24.1	+		
" " "	5.0	0.20		23.8	16.5		
" " "	5.4	0.20		23.8	16.1		
KH_2PO_4, Na_2HPO_4, NaCl.	6.0	0.20		23.7	16.6		
" " " .	6.8	0.20		23.2	+		
" " " .	7.5	0.20		22.5	+		
KH_2PO_4, $Na_2B_4O_7$, NaCl..	8.0	0.25		22.7	+		
" " " ..	8.5	0.25		23.1	+		
Na_2CO_3, $Na_2B_4O_7$, NaCl..	9.0	0.25		22.1	+		
" " " ..	9.5	0.25		23.7	17.5		
" " " ..	10.0	0.25		24.3	17.7		
" " " ..	10.5	0.25		22.7	15.7		
" " " ..	10.5	0.25		23.0	+		
Na_2HPO_4, NaOH, NaCl..	11.0	0.30		23.9	16.2	4.60	Inhomogeneous
" " " ..	11.5	0.30				4.82	"
			32.7	23.6	16.4	4.71	

* Not used for calculation of mean value.

pH 11.5 G and H have disappeared. K is not quite homogeneous. At pH 3.0 the hemocyanin is aggregated and non-uniform. Figure 9 gives an example of a sedimentation run and Fig. 10 (Plate I) the pH-stability diagram.

TABLE VII

Carcinus mænas

Dilution of blood 6 times; centrifugal force 175,000 (speed 50,000 r.p.m.); in the last two runs 280,000 (speed 63,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	s_{20} K	
HAc, NaAc, NaCl	3.6	0.20	23.6	17.1		
" " "	4.0	0.20	24.0	17.1		
" " "	4.6	0.20	23.9	16.8		
" " "	5.0	0.35	22.5	15.7		
KH_2PO_4, Na_2HPO_4, NaCl	6.0	0.35	23.0	16.5		
" " KCl	6.8	0.12	23.5	16.2		
" " NaCl	7.4	0.20	22.6	+		
" " KCl	8.0	0.12	23.4	16.6		
" $Na_2B_4O_7$, NaCl	8.5	0.25	23.7	16.3		
" " "	9.0	0.25	23.7	17.3		
" " "	9.5	0.25	23.1	17.3		
" " "	10.0	0.25	22.7	16.7		
" " "	10.5	0.25	22.5	14.5*		
NaOH " "	11.2	0.27			5.00	Inhomogeneous
" " "	11.5	0.30			4.17	Inhomogeneous
			23.3	16.7		

* Not used for calculation of mean value.

Carcinus mænas

Kristineberg, Sweden

The sedimentation constant of the main component G is 23.3. It is stable from pH 3.6–10.5. A smaller component with the sedimentation constant 16.7 is present throughout the same range but to a very small amount. At pH 11.2 both are dissociated into component K, a low-molecular inhomogeneous split-product. Figure 11 (Plate I) gives the pH-stability diagram.

Arachnomorpha: Xiphosura:

Limulus polyphemus

Woods Hole, Mass., U. S. A.

In the blood four different components are present at the same time in the pH-range 5.2–10.5. Their sedimentation constants are 56.6 (D), 34.6 (F), 16.1 (H), and 5.87 (K). The proportions between their concentrations are unchanged inside this region. Above 10.5 D, F, and H disappear; they split up into K. Below pH 5.2 D and F dissociate in two ways. The concentration of K apparently increases; *i.e.*, some part of them must form more of K. A new component, G, with the sedimentation constant 24.0 appears in a rather high concentration; and G must be a dissociation product of D and F as the concentration of H does not change and the concentration of K increases.

TABLE VIII

Limulus polyphemus

Dilution of blood 4 times; centrifugal force 125,000 (speed 41,000 r.p.m.); in the last three runs 300,000 (speed 65,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} D	s_{20} F	s_{20} G	s_{20} H	s_{20} K	
HAc, NaAc, NaCl......	3.6	0.36			24.2	16.2	6.02	
" " "	4.0	0.19			23.5	16.1	6.15	
" " "	4.2	0.36			23.5	16.4	5.24	
" " "	4.4	0.19			23.3	16.2	+	
" " "	4.6	0.19	55.2*		23.5	15.8	+	
" " "	4.6	0.19			24.5	16.5	+	
" " "	4.8	0.19		35.2	23.1	15.8	+	
" " "	4.8	0.19			24.7	17.0	+	
" " "	5.0	0.19			25.8	16.8	6.3	
" " "	5.2	0.19	56.3	34.8		16.1	+	
KH_2PO_4, Na_2HPO_4, NaCl	5.4	0.24	58.2	35.3		17.2	+	
" " "	5.6	0.19	59.7	38.1		16.2	+	
" " "	6.3	0.36	56.5	36.7		15.5	+	
" " "	6.3	0.36	+	+		15.5	5.0†	
" " "	6.8	0.19	57.3	36.4		16.3	+	
" " "	7.2	0.36	57.1	35.1		15.5	+	
" " "	7.6	0.19	56.4	36.0		15.9	+	
" " "	8.0	0.36	58.0	36.0		16.0	+	
Na_2HPO_4, NaCl........	8.9	0.20	58.2	33.8		16.1	6.28	
" NaOH, NaCl .	9.8	0.22	56.1	35.1		15.8	+	
" " " .	10.2	0.22	52.6	30.0		17.6	6.25	
Na_2CO_3, $Na_2B_4O_7$, NaCl .	10.3	0.22	53.4	29.6		14.4	5.70	
" " " .	10.5	0.23	54.6	32.3		16.1	5.86	
Na_2HPO_4, NaOH, NaCl .	10.7	0.23					5.14	
Na_2CO_3, $Na_2B_4O_7$, NaCl .	10.8	0.23				+	4.88*	
Na_2HPO_4, NaOH, NaCl .	11.5	0.27					4.45*	Dialysed blood
Na_2HPO_4, NaOH, NaCl .	12.1	0.27					4.26*	Dialysed blood
			56.5	34.6	24.0	16.1	5.87	

* Not used for the calculation of mean value.

† Made at 60,000 r.p.m. and with blood diluted 2 times to get component K.

Figure 12 gives an example of a sedimentation run and Fig. 13 (Plate I) the pH-stability diagram.

Mollusca

The bloods of the Amphineura, the Gastropoda and the Cephalopoda contain hemocyanin. Because of the difficulty of obtaining sufficient material from specimens of the first class our investigation has been limited to species belonging to the two latter classes.

Conchifera: Gastropoda:

Blood from the following animals belonging to this group has been investigated: *Littorina littorea*, *Neptunea antiqua*, *Buccinum undatum*, *Busycon canaliculatum*, *Helix pomatia*, *Helix arbustorum*, *Helix nemoralis*, *Helix hortensis*, *Limax maximus*. They show extremely varied stability diagrams and a great number of dissociation products. The main component has a sedimentation constant of about 100. In blood from two of the animals, *Busycon* and *Limax*, a higher component with the sedimentation constant near 130 is found. All, except *Neptunea*, show a dissociation product with a sedimentation constant of about 60. Very often a component with sedimentation constant of approximately 16 is found and the final alkaline dissociation products have sedimentation constants around 11. It is also striking that the four kinds of *Helix* have different stability diagrams, so that each of them could be characterized thereby.

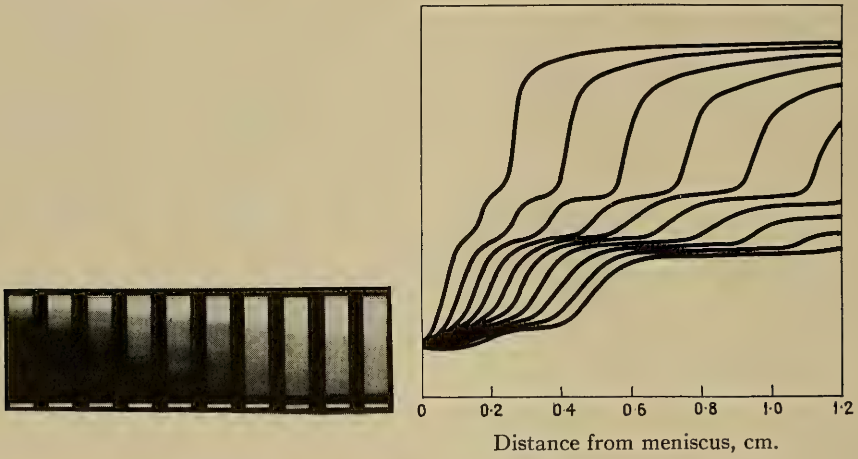


FIG. 12*a*.

FIG. 12*b*.

FIG. 12. Sedimentation pictures (*a*) with photometric records (*b*) for the hemocyanin from *Limulus polyphemus* at pH 6.8 (s_{20} = 56.6, 34.6, 16.1, and 5.87); centrifugal force 120,000; time between exposures 5 minutes.

Littorina littorea

Kristineberg, Sweden

Between pH 5 and 8.5 this hemocyanin shows one homogeneous component B, with the sedimentation constant 99.7. At pH 8.5 B is partly split up into components C and H. In the pH-range 8.5–10.7 most of the hemocyanin is present in form of C, with the sedimentation constant 63.3. H appears only in very small amount up to pH 10.8 where most of B and C are dissociated into H and above it is the only component present. When first observed H has a sedimentation constant of about 15, but it decreases towards the alkaline side and the protein becomes inhomogeneous. At pH 4.0 precipitation takes place. Figure 14 (Plate II) gives the pH-stability diagram.

TABLE IX

Littorina littorea

Dilution of blood 4 times; centrifugal force 49,000 (speed 25,000 r.p.m.); in the last three runs 180,000 (speed 50,000 r.p.m.); thickness of column of solution, 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	
NaAc, HAc, NaCl......	4.0	0.22				Solubility too low for determination
" " "	5.0	0.22	96.9			
" " "	5.5	0.22	99.7			
Na_2HPO_4, KH_2PO_4, NaCl	5.9	0.22	99.8			
" " "	6.2	0.22	98.4			
" " "	6.8	0.22	98.5			
" " "	7.4	0.22	97.0			
" " "	8.0	0.22	94.6			
$Na_2B_4O_7$, " "	8.5	0.27	102.1	63.5	+	
" " "	9.0	0.27	102.1	64.8	+	
" Na_2CO_3, "	9.5	0.25	101.4	63.0	+	
" " "	10.0	0.25	100.0	62.2	+	
" " "	10.3	0.25	97.9	62.5	+	
" " "	10.5	0.25	104.6	64.3	+	
" " "	10.6	0.25	102.0	62.3	+	
" " "	10.7	0.25	102.9	63.4	+	
" " "	10.8	0.25	97.3	59.5*	14.5	
Na_2HPO_4, NaOH, "	11.1	0.25			15.6	
" " "	11.3	0.25			11.3*	
" " "	11.5	0.25			12.9*	
			99.7	63.3	15.0	

* Not used for the calculation of mean value.

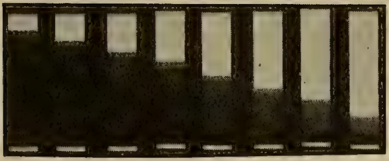


FIG. 15*a*.

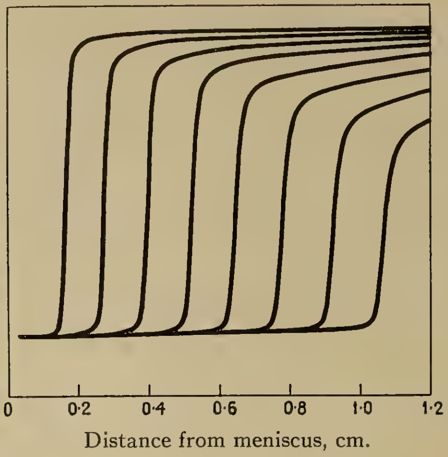


FIG. 15*b*.

FIG. 15. Sedimentation pictures (*a*) with photometric records (*b*) for the hemocyanin from *Neptunea antiqua* at pH 8.0 (s_{20} = 104.1); centrifugal force 40,000; time between exposures 5 minutes.

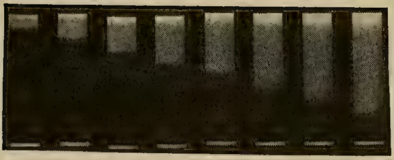


FIG. 16*a*.

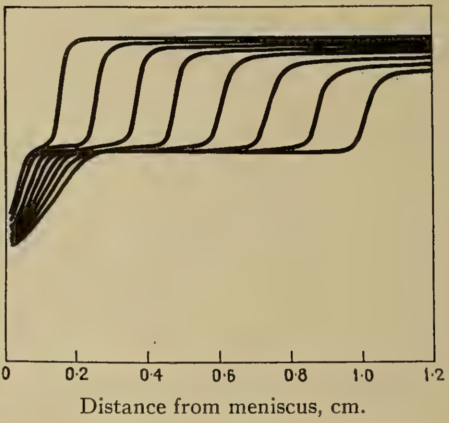


FIG. 16*b*.

FIG. 16. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Neptunea antiqua* at pH 8.6 (s_{20} = 104.1 and 14.3); centrifugal force 60,000; time between exposures 5 minutes.

Neptunea antiqua

Kristineberg, Sweden

In the pH-range 4.6–8.6 the hemocyanin is homogeneous and shows one component, B, the sedimentation constant of which is 104.1. At 8.6 there begins a partial splitting into component H with the sedimentation constant 14.3. In the pH-region 8.6–10.0 B and H are present together. At pH 10.8 the splitting is total, B disappears and a new component, I, with a sedimentation constant near 13 appears. The sedimentation constant of I decreases at a higher pH and the protein becomes inhomogeneous. In acid solutions aggregation takes place. The material becomes more and more inhomogeneous, the particles larger. At pH 3.0 most of the protein is precipitated. Figures 15 and 16 give examples of sedimentation runs and Fig. 17 (Plate II) the pH-stability diagram.

TABLE X

Neptunea antiqua

Dilution of blood 15–20 times; centrifugal force 49,000 (speed 25,000 r.p.m.); in the last three runs 180,000 (speed 50,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} H	s_{20} I	
NaAc, HAc, NaCl........	3.0	0.19				Most of the material precipitated
“ “ “	3.6	0.19	116.8*			Inhomogeneous
“ “ “	4.0	0.19	138.2*			“
“ “ “	4.2	0.19	110.6*			“
“ “ “	4.6	0.19	102.4			
“ “ “	5.0	0.19	104.7			
Na_2HPO_4, KH_2PO_4, NaCl .	5.5	0.19	102.7			
“ “ “ .	6.0	0.19	101.1			
“ “ KCl ..	6.8	0.14	106.7			
“ “ NaCl .	7.1	0.19	102.8			
“ “ “ .	7.5	0.19	105.6			
“ “ “ .	8.0	0.19	106.9			
$Na_2B_4O_7$, “ “ .	8.6	0.20	100.7	14.0		
“ “ “ .	8.8	0.20	104.0	11.6		
“ “ “ .	9.0	0.20	101.4	13.6		
“ Na_2CO_3, “ .	9.25	0.20	107.8	+		
“ “ “ .	9.5	0.20	102.8	+		
“ “ “ .	10.0	0.20	107.0	15.9		
“ “ “ .	10.8	0.20		15.7	13.8	Inhomogeneous
“ “ “ .	10.8	0.20		15.1	13.3	“
NaOH, Na_2HPO_4, “ .	12.1	0.27			9.48	“
			104.0	14.3		

* Not used for calculation of mean value.

Buccinum undatum

Kristineberg, Sweden

The main component, B, of this hemocyanin is observed in the pH-range 5–10.8. Its sedimentation constant is 102.1. B is present alone from pH 5 to 9.5. Here a very small quantity of C, a component with sedimentation constant 63.8 is formed. The relative amount of C increases with pH. Above 10.8 B and C are dissociated into more inhomogeneous material with a sedimentation constant around 11. At pH 4.0 the hemocyanin is precipitated. Figure 18 (Plate II) gives the pH-stability curve.

TABLE XI

Buccinum undatum

Dilution of blood 7 times; centrifugal force 49,000 (speed 25,000 r.p.m.); in the last four runs 220,000 (speed 55,000 r.p.m.); other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} I	
NaCl. .		0.17	104.5			
	4.0					Protein precipitated
HAc, NaAc, " . .	5.0	0.20	104.0			
KH_2PO_4, Na_2HPO_4, " . .	5.9	0.20	98.4			
" " " . .	6.2	0.20	100.9			
" " KCl. . .	6.8	0.12	102.7			
" " NaCl. .	7.4	0.20	100.7			
" $Na_2B_4O_7$, KCl. . .	8.0	0.12	103.9			
" " NaCl. .	8.5	0.25	103.2			
" " " . .	9.0	0.25	99.6			
Na_2CO_3, " " . .	9.5	0.25	101.5			
" " " . .	10.0	0.25	105.6	64.6		
" " " . .	10.8	0.25	100.5	63.1	+	
" " " . .	10.8	0.25			12.8	
NaOH, Na_2HPO_4, " . .	11.3	0.30			10.5	Very inhomogeneous
" " " . .	11.5	0.30			11.0	" "
" " " . .	12.1	0.35			9.2	" "
			102.1	63.8		

Busycon canaliculatum

Woods Hole, Mass., U.S.A.

The hemocyanin from this animal shows a very complicated stability diagram. Four well-defined components, A, B, C, and H are observed within the investigated pH-range 3–12. The sedimentation constants are: for A 130.4, for B 101.7, for C 61.1 and for H 13.5. A and B are present together in the region 4.5–7.7. The relative amount of A is larger towards the acid side. It is not perfectly homogeneous; the sedimentation curves have a shape indicating the presence of slightly faster sedimenting particles. At 4.0 the protein aggregates, and becomes inhomogeneous. In the region 7.7–9.0 there are three components present, B, C and H. The amount of B is larger in the less alkaline part of the region, at 9.0 it is only several per cent of the whole protein. The percentages of C and H increase with pH. In the pH-region 9.0–10.8 the components C and H are found. Above 10.8 only H is left and it there becomes more and more inhomogeneous. Figures 19 and 20 give examples of sedimentation runs and Fig. 21 (Plate II) the pH-stability curve.

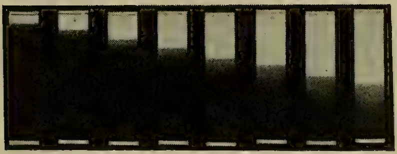


FIG. 19*a*.

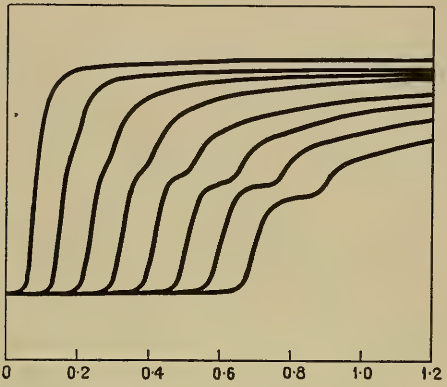


FIG. 19*b*.

FIG. 19. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Busycon canaliculatum* at pH 6.5 (s_{20} = 130.4 and 101.7); centrifugal force 40,000; time between exposures 5 minutes.

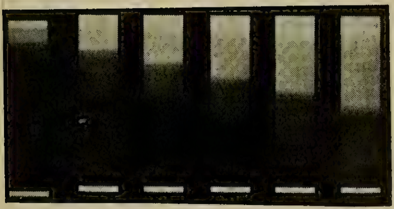


FIG. 20*a*.

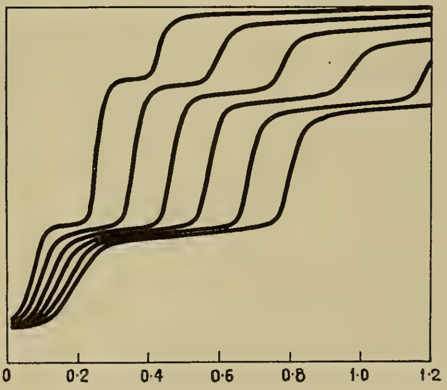
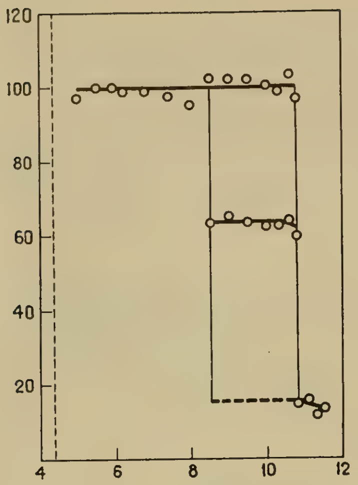


FIG. 20*b*.

FIG. 20. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Busycon canaliculatum* at pH 8.5 (s_{20} = 101.7, 61.1, and 13.5); centrifugal force 70,000; time between exposures 5 minutes.



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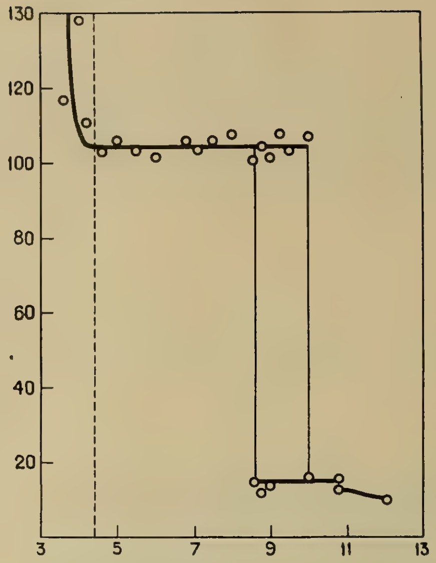

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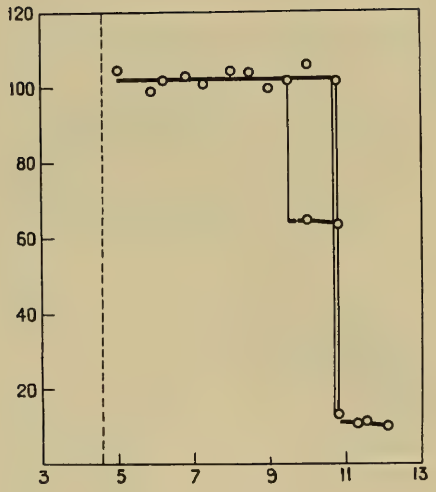

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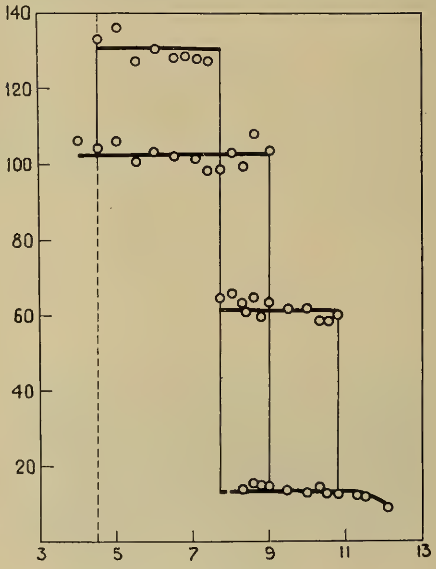

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PLATE II

pH-stability diagrams for hemocyanins of species listed below. Abscissæ in all figures, pH; ordinates, s_{20}. The dotted lines indicate the positions of the isoelectric points.

FIG. 14. *Littorina littorea.*
FIG. 17. *Neptunea antiqua.*
FIG. 18. *Buccinum undatum.*
FIG. 21. *Busycon canaliculatum.*

Helix pomatia

Upsala, Sweden

The hemocyanin appears in four different well-defined molecular species, B with the sedimentation constant 98.9, C with 62.0, H with 16.0, and I with 12.1. The main component is B, present in solutions with a pH from 3.6 to 8.2. It is alone in the pH-region 4.6–7.4 and accompanied by C in the regions 3.6–4.6 and 7.4–8.2. H is present in very small quantity together with B and C in the pH-range 7.9–8.2,

TABLE XII

Busycon canaliculatum

Dilution of blood 20 times, centrifugal force in the pH-range 3–8, 35,000 (speed 23,000 r.p.m.), in the pH-range 8–11, 60,000 (speed 30,000 r.p.m.), above pH 11, 200,000 (speed 55,000 r.p.m.); thickness of column of solution, 0.6 cm., other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} A	s_{20} B	s_{20} C	s_{20} I	
HCl, Na-citrate, NaCl...	3.0	0.30		45.5			Inhomogeneous
HAc, NaAc, NaCl.......	3.4–3.6	0.19					Solubility too low for determination
" " "	4.0	0.19		106.0			
" " "	4.5	0.19	133.0	103.8			
" " "	5.0	0.19	137.0	107.2			
" " "	5.0	0.19	134.9	104.6			
KH_2PO_4, Na_2HPO_4, NaCl	5.5	0.19	126.8	100.2			
" " "	6.0	0.19	130.1	102.8			
" " "	6.5	0.19	128.1	102.1			
" " KCl .	6.8	0.12	128.5	102.0			
" " NaCl	7.1	0.19	127.7	101.7			
" " "	7.4	0.19	127.3	98.3			
" " "	7.7	0.19		98.6	64.1	+	
" " KCl .	8.0	0.12		102.9	65.5	+	
" $Na_2B_4O_7$, NaCl	8.3	0.23		98.9	62.7	+	
" " "	8.4	0.23		96.1	59.6	12.2	
" " "	8.4	0.23		102.2	60.3	14.1	
" " "	8.5	0.23		97.1	60.0	14.2	
" " "	8.6	0.23		107.4	64.0	14.9	
Na_2CO_3, " "	9.0	0.23		103.3	61.7	14.7	
" " "	9.5	0.23			60.9	13.0	
" " "	10.0	0.23			60.5	13.0	
" " "	10.3	0.23			58.0	14.0	
" " "	10.5	0.23			58.2	12.6	
" " "	10.8	0.23			58.5	13.6	
Na_2PHO_4, NaOH, "	11.3	0.27				12.0	
" " "	11.5	0.30				11.5*	Inhomogeneous
" " "	12.1	0.30				8.52*	"
			130.4	101.7	61.1	13.5	

* Not used for calculation of mean value.

TABLE XIII

Helix pomatia

Dilution of blood 15 times; centrifugal force in the range pH 3–8, 50,000 (speed 27,000 r.p.m.); above pH 8 150,000 (speed 45,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	s_{20} I	
HCl, Na-citrate, NaCl. .	3.3	0.27					22.5 Inhomogeneous
HAc, NaAc, NaCl.	3.6	0.20	98.2	61.9			
“ “ “	3.6	0.37		60.5			26.5 “
“ “ “	3.8	0.37	102.5	63.1			
“ “ “	3.8	0.20	95.6	59.2			
“ “ “	4.0	0.20	100.7	59.3			
“ “ “	4.0	0.20	97.6	62.6			
“ “ “	4.2	0.20	103.1	61.8			
“ “ “	4.2	0.37	102.6	66.2			
“ “ “	4.6	0.20	95.9	+			
“ “ “	5.0	0.20	102.2				
“ “ “	5.4	0.20	99.8				
KH_2PO_4, Na_2HPO_4, NaCl	5.5	0.20	102.5				
“ “ “	6.0	0.20	94.8				
“ “ “	6.4	0.20	102.1				
“ “ “	7.0	0.20	98.3				
“ “ “	7.4	0.20	94.3	+			
“ “ “	7.5	0.20	89.6*	+			Bad separation of the 2 components
“ “ “	7.6	0.20	102.6	56.7			
“ “ “	7.7	0.20	97.4	64.2			
“ “ “	7.9	0.20	96.5	63.0			
“ “ “	8.0	0.20	97.3	62.5	+		
“ $Na_2B_4O_7$, “	8.1	0.23	94.1	65.8			
“ “ “	8.2	0.23	99.3	62.8	19.5*		
“ “ “	8.3	0.23			16.5		
“ “ “	8.4	0.24			15.3		
“ “ “	8.5	0.24			16.5		
“ “ “	8.6	0.24			16.6		
“ “ “	8.9	0.24			17.7		
“ “ “	9.3	0.24			15.7	+	
Na_2CO_3, “ “	9.4	0.22			15.8	13.4	
“ “ “	9.5	0.23			15.7	11.9	
“ “ “	9.7	0.23			15.4	+	
“ “ “	9.8	0.22			14.8		
“ “ “	9.8	0.23			14.7	11.9	
“ “ “	9.8	0.23			15.6	11.1	
“ “ “	10.0	0.23			18.1	+	
“ “ “	10.3	0.23			15.0	11.5	
“ “ “	10.5	0.23			15.1	11.7	
“ “ “	10.6	0.42			18.1	13.9	
“ “ “	10.8	0.25			+	11.3	
NaOH, Na_2HPO_4, “	11.5	0.30				9.9*	Inhomogeneous
“ “ “	12.1	0.30				8.4*	“
			98.9	62.0	16.0	12.1	

* Not used for the calculation of mean value.

alone between pH 8.2 and 9.3, and together with I in the pH-range 9.3–10.8. In more alkaline solutions the hemocyanin is inhomogeneous and the sedimentation constant decreases. Below pH 3.6 the material is non-uniform and the sedimentation constants are irregular but apparently decreasing. Figure 22 (Plate III) gives the pH-stability diagram.

Table XIV

Helix arbustorum

Dilution of blood 5 to 30 times; centrifugal force in the pH-region 3–8, 50,000 (speed 26,000 r.p.m.); above pH 8 175,000 (speed 50,000 r.p.m.); thickness of column of solution 0.4–2.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	s_{20} I	
HCl, Na-citrate, NaCl....	2.5	0.20					Very inhomogeneous
" " "	2.9	0.20			20.9	12.5	" "
HAc, NaAc, NaCl.......	3.6	0.20	83.8	56.0			
" " "	4.0	0.20	94.2	56.5			
" " "	4.6	0.20	89.7	71.5			
" " "	5.0	0.20	95.4	+			
" " "	5.0	0.20	87.8	59.8			
KH_2PO_4, Na_2HPO_4, NaCl.	5.6	0.20	97.0	74.1			
" " " .	6.0	0.20	92.2	65.2			
" " " .	6.3	0.20	90.0	61.9			
" " " .	6.5	0.20	90.1	68.9			
" " " .	6.8	0.20	88.3	63.0			
" " " .	7.0	0.20	90.8	+			
" " " .	7.4	0.20	93.1	66.5			
" " " .	7.7	0.20	96.8	60.9			
" " " .	8.0	0.20	87.2	+			
" $Na_2B_4O_7$, NaCl..	8.2	0.25			16.1		
" " " ..	8.5	0.25			16.1		
" " " ..	8.7	0.25			15.6	+	
Na_2CO_3, " " ..	9.0				16.4	13.0	
" " " ..	9.4				14.9	11.0	
" " " ..	9.5				16.8	12.5	
" " " ..	10.14	0.25			15.5	11.9	
" " " ..	10.3				14.0	10.9	
" " " ..	10.6				10.9	9.7	
Na_2HPO_4, NaOH, NaCl..	10.8	0.27			15.7	11.3	
" " " ..	11.0	0.32			13.7	10.4	
" " " ..	11.3	0.27			14.6	9.3	
			91.2	64.1	15.0	11.1	

Helix arbustorum

Upsala, Sweden

The hemocyanin has two components, B and C, in the pH-range 3.6–8.0. The two components seem to influence each other in a way that makes accurate determination of their sedimentation constants rather difficult. The mean value for B is 91.2 and for C 64.1.

The proportions between B and C change in an irregular way.

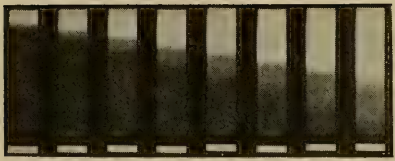


FIG. 23*a*.

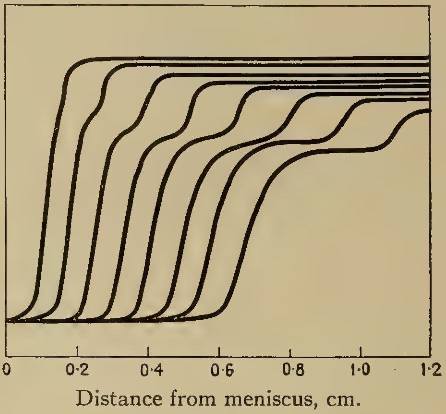


FIG. 23*b*.

FIG. 23. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Helix arbustorum* at pH 7.7 (s_{20} = 91.2 and 64.1); centrifugal force 50,000; time between exposures 5 minutes.

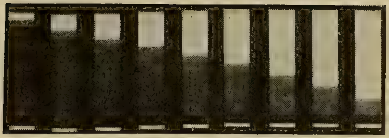


FIG. 25*a*.

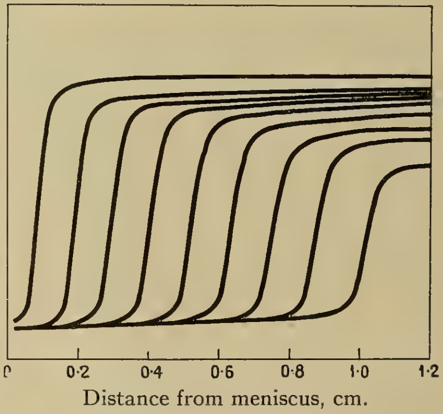


FIG. 25*b*.

FIG. 25. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Helix nemoralis* at pH 8.5 (s_{20} = 16.6); centrifugal force 125,000; time between exposures 5 minutes.

Some runs indicate that in more dilute solutions the amount of C increases. At pH 8.2 B and C are dissociated into a uniform component H with sedimentation constant 15.0. H is the single molecular species present in the pH-region 8.0–8.7. At pH 8.7 a small part of it is split up into a fourth component I with the sedimentation constant

11.1. H and I are present together in the entire pH-region investigated above pH 8.7. The amount of I increases with pH. At pH 2.9 and below the hemocyanin is inhomogeneous. Figure 23 gives an example of a sedimentation run and Fig. 24 (Plate III) the pH-stability curve.

Helix nemoralis

Upsala, Sweden

The main component, B, of this hemocyanin, has the sedimentation constant 101.0. It is present in the pH-range 3.6–8.0. Between pH 3.6 and 4.2 it is accompanied by component C with the sedimentation constant 65.0. At pH 7.7 and 8.0 there are also traces of C. Above pH 8 B and C are split up into the very homogeneous dissociation product H with the sedimentation constant 16.6. H is present alone up to 10.5 where I with the sedimentation constant 11.9 appears. H and I coexist to 10.8. In the region 10.8–11.5 I is alone. At 11.5 another dissociation product, still lower, is present together with I. Around pH 3 the material is rather inhomogeneous. At pH 2.5 H and I are formed. Figure 25 gives an example of a sedimentation run and Fig. 26 (Plate III) the pH-stability curve.

Helix hortensis

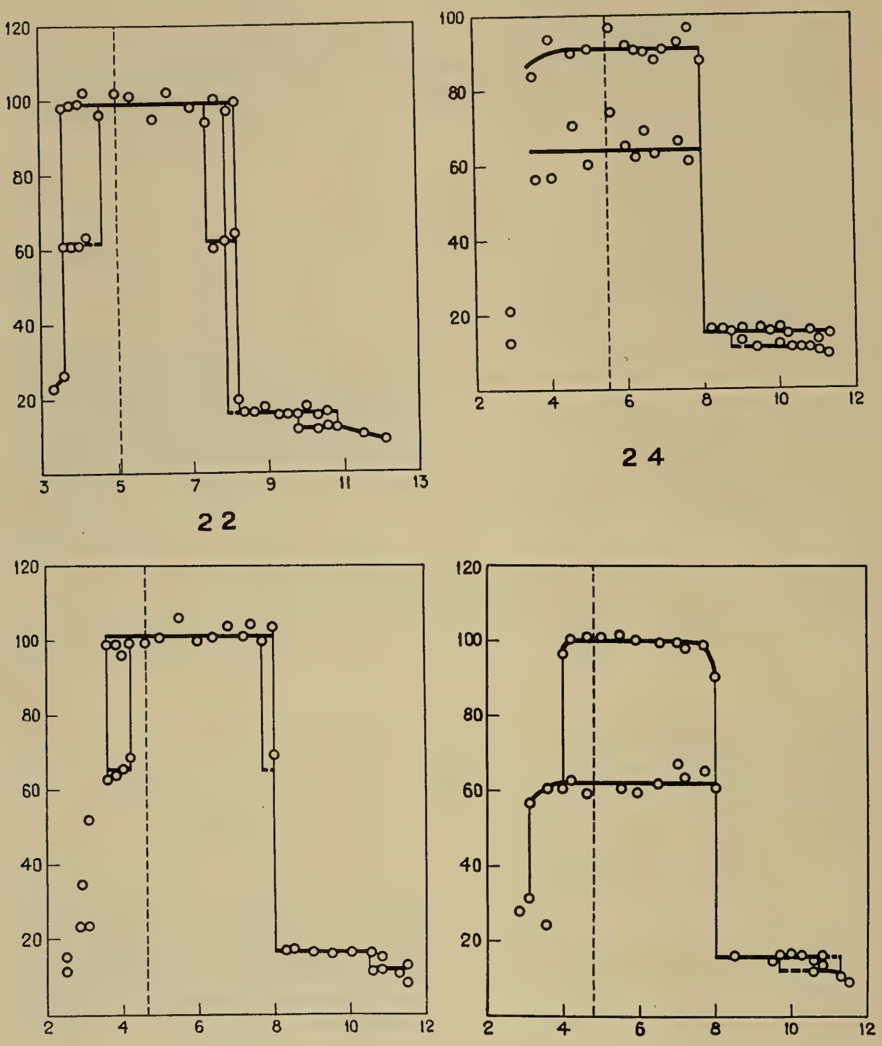
Upsala, Sweden

In the pH-region 4.0–8.0 this hemocyanin has two components, B with the sedimentation constant 100.0 and C with the sedimentation constant 61.9. B is the main component, C only forms 15 per cent of the whole protein up to pH 7.2. Between 7.2 and 8.0 B is present in a higher relative amount. In the pH-range 8–9.7 the material is split up into component H with the sedimentation constant 15.9. At 9.7 the splitting goes further, beside H component I with the sedimentation constant 12.2 is present. At pH 11.3 and above H disappears. In the region 3.6–4.0 C is present alone and in more acid solutions the hemocyanin is split up into inhomogeneous, more low-molecular dissociation products. Figure 27 (Plate III) gives the stability diagram.

Limax maximus

Värmdön, Sweden

The main component, B, has the sedimentation constant 97.3. It is present in the pH-range 4.0–8.0 together with a higher component, A. A is rather inhomogeneous and low in concentration, therefore the determination of its sedimentation constant is uncertain. As mean value 136 has been found. At pH 4.0–4.2 a lower component C with



22

24

26

27

PLATE III

pH-stability diagrams for hemocyanins of species listed below. Abscissæ in all figures, pH; ordinates, s_{20}. The dotted lines indicate the positions of the isoelectric points.

FIG. 22. *Helix pomatia.*
FIG. 24. *Helix arbustorum.*
FIG. 26. *Helix nemoralis.*
FIG. 27. *Helix hortensis.*

the sedimentation constant 61.4 appears. At pH 3.6–3.8 the hemocyanin is further dissociated into three components, G, H, and I, with the sedimentation constants 25.5, 18.0, and 13.7. Above pH 8 A and B are dissociated into component H. Figure 28 (Plate IV) gives the pH-stability diagram.

TABLE XV

Helix nemoralis

Dilution of blood 10 times; centrifugal force in the pH-range 2–8 50,000 (speed 26,000 r.p.m.); above pH 8 210,000 (speed 54,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	s_{20} I	
HCl, Na-citrate, NaCl. . .	2.5	0.30			15.0	11.3	
" " " . . .	2.9	0.30					34.7, 23.2 inhomogeneous
" " " . . .	3.1	0.30					52.3, 23.7 inhomogeneous
HAc, NaAc, NaCl.	3.6	0.20	98.8	62.7			
" " "	3.8	0.20	99.3	63.4			
" " "	4.0	0.20	96.9	65.5			
" " "	4.2	0.20	98.9	68.4			
" " "	4.3	0.20	99.5				
" " "	4.6	0.20	99.1				
KH_2PO_4, Na_2HPO_4, NaCl	5.0	0.20	100.4				
" " "	5.5	0.20	104.4				
" " "	5.6	0.20	107.7				
" " "	6.0	0.20	99.5				
" " "	6.4	0.20	100.7				
" " "	6.8	0.20	103.4				
" " "	7.2	0.20	101.0				
" " "	7.4	0.20	103.7				
" " "	7.7	0.20	100.1	+			
" " "	7.7	0.20	99.5	+			
" " KCl .	8.0	0.12	104.9	69.4			
" $Na_2B_4O_7$, NaCl .	8.3	0.12			16.5		
" " " .	8.5	0.25			16.8		
" " " .	9.0	0.25			16.6		
Na_2CO_3, $Na_2B_4O_7$, NaCl. .	9.5	0.25			16.2		
" " " . .	10.0	0.25			16.4		
" " " . .	10.5	0.25			16.9	+	
" " " . .	10.6	0.25			15.2	11.6	
" " " . .	10.8	0.25			15.1	11.8	
NaOH, Na_2HPO_4, NaCl. .	11.3	0.30				10.5	
" " " . .	11.5	0.30				12.7	9.0
" " " . .	11.5	0.30				12.8	8.5
			101.0	65.0	16.6	11.9	

Conchifera: Cephalopoda:

Decapoda. Hemocyanins from three animals belonging to this group: *Sepia officinalis*, *Rossia owenii*, and *Loligo vulgaris*, have been investigated. The main component of their blood has the sedimentation constants 55.9, 56.2, and 56.7 respectively. They are identical within limits of experimental error. Moreover, their final alkaline dissociation products are the same. *Sepia* and *Loligo* have components with the sedimentation constants 18.7 and 16.9. These probably represent a splitting of the original molecule into the same number of

TABLE XVI

Helix hortensis

All conditions as in Table XIV

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	s_{20} I	
Na-citrate, HCl, NaCl...	2.5	0.20					Very inhomogeneous
" " " ...	2.9	0.30					28.1, 19.3, 13.0 Very inhomogeneous
" " " ...	3.1	0.30		56.7*			31.1 inhomogeneous
HAc, NaAc, NaCl.......	3.6	0.20		60.7			24.0 inhomogeneous
" " "	4.0	0.20	96.5	60.3			
" " "	4.2	0.20	100.5	62.8			
" " "	4.6	0.20	101.1	58.9			
" " "	5.0	0.20	100.9	+			
KH_2PO_4, Na_2HPO_4, NaCl	5.5	0.20	101.5	60.8			
" " "	5.9	0.20	100.6	59.3			
" " "	6.5	0.20	100.1	61.4			
" " "	6.6	0.20	98.6	62.0			
" " "	7.0	0.20	99.5	66.7			
" " "	7.2	0.20	98.0	63.4			
" " "	7.7	0.20	99.1	65.4			
" " KCl .	8.0	0.12	+	+			
" $Na_2B_4O_7$, NaCl..	8.5	0.23			16.6		
Na_2CO_3, $Na_2B_4O_7$, NaCl..	9.5	0.23			14.8		
" " " ..	9.7	0.20			16.5	+	
" " " ..	10.0	0.23			16.5	+	
" " " ..	10.3	0.23			16.3	+	
" " " ..	10.6	0.20			14.7	12.3	
" " " ..	10.8	0.23			15.8	13.8	
NaOH, Na_2HPO_4, NaCl..	11.3	0.30			+	10.3	
" " " ..	11.5	0.30				8.99*	
			100.0	61.9	15.9	12.1	

* Not used for the calculation of mean value.

fragments. In all three of them there is observed an inhomogeneous component of rather high sedimentation constant which exists in a narrow range inside the pH-region where the main component is stable.

Octopoda. This group is represented by two animals, *Octopus vulgaris* and *Eledone moschata.* The normal sedimentation constants for the hemocyanin in their blood are 49.3 for *Octopus* and 49.1 for *Eledone.* Only one dissociation product is found and this is identical with the final one in the blood from the decapods. The molecular weight of this small component probably represents 1/7 of the normal molecular weight of the octopod hemocyanin and 1/8 of the molecular weight of the decapod hemocyanin.

TABLE XVII

Limax maximus

Dilution of blood 10 times; centrifugal force in the pH-range 3.6–8.0 50,000 (speed 26,000 r.p.m.); above 8.0 175,000 (speed 50,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} A	s_{20} B	s_{20} C	s_{20} G	s_{20} H	s_{20} I
HAc, NaAc, NaCl............	3.6	0.20				24.3	18.6	13.7
" " "	3.8	0.20				26.6	19.9	13.6
" " "	4.0	0.20	+	95.2	60.8			
" " "	4.2	0.20	143.0	96.3	63.0			
" " "	4.6	0.20	+	100.7				
" " "	5.0	0.20	126.3	97.0				
" " "	5.0	0.20	+	100.5				
KH_2PO_4, Na_2HPO_4, NaCl......	5.6	0.20	+	97.8				
" " "	5.9	0.20	128.9	93.0				
" " "	6.4	0.20	129.1	93.2				
" " KCl.......	6.8	0.20	+	99.3				
" " NaCl......	2.2	0.20	153.1	92.4				
" " "	7.4	0.20	+	96.1				
" " "	8.0	0.20	+	105.6				
" $Na_2B_4O_7$, NaCl.......	8.5	0.25					17.8	
" " "	9.0	0.25					17.1	
Na_2CO_3, $Na_2B_4O_7$, NaCl.......	10.0	0.25					16.6	
			136.0	97.3	61.9	25.5	18.0	13.7

Loligo vulgaris

Naples, Italy

The hemocyanin appears in four different molecular species, namely, the components D, F, H, and I. Of those D, H, and I are well-defined and homogeneous; they give the sedimentation constants 56.7, 16.9, and 12.1. F is very inhomogeneous and it appears irregularly. The mean value of its sedimentation constant is 35.9.

Component D exists in the pH-range 5.0–10.0 with the exception of a narrow region around 8. It is present alone between pH 5.0 and 7.2, accompanied by H from pH 7.2 to 10.0 and by F from 7.7 to 8.7. Component I appears together with D and H in the pH-range 9.6–10.0 and is the single component present above 10.0. Above pH 11 it is still perfectly homogeneous with regard to molecular weight but its sedimentation constant drops gradually. This must be due to a change in shape of the particles. Below pH 5 aggregation sets in. Figures 29 and 30 give examples of sedimentation runs, Fig. 31 (Plate IV) gives the pH-stability curve.

TABLE XVIII

Loligo vulgaris

Dilution of blood 10 times; centrifugal force in the pH-range 5–10 60,000 (speed 30,000 r.p.m.); above pH 10 260,000 (speed 60,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	S_{20} D	S_{20} F	S_{20} H	S_{20} I
HAc, NaAc, NaCl................	5.0	0.20	55.6			
KH_2PO_4, Na_2HPO_4, NaCl.........	5.5	0.20	55.9			
" " "	5.9	0.20	56.3			
" " "	6.5	0.20	56.9			
" " "	6.8	0.20	54.1			
" " "	7.2	0.20	58.9		21.2	
" " "	7.7	0.20	57.9	39.3		
" " "	8.0	0.20		+	18.9	
" " "	8.0	0.20		31.5	18.3	
" $Na_2B_4O_7$, "	8.4	0.25	57.8	+	+	
" " "	8.7	0.25	57.1	37.0	16.6	
" " "	9.2	0.25	57.5		14.0	
Na_2CO_3, " "	9.4	0.25	57.6		17.5	
" " "	9.6	0.25	55.3		16.9	12.6
" " "	9.8	0.25	+		16.2	12.5
" " "	10.0	0.25	56.6		+	+
" " "	10.3	0.25				12.0
" " "	10.6	0.25				11.4
" " "	11.1	0.27				10.5
" " "	11.5	0.30				9.4*
" " "	11.7	0.35				9.0*
			56.7	35.9	16.9	12.1

* Not used for the calculation of mean value.

Rossia owenii

Kristineberg, Sweden

The main component, D, has the sedimentation constant 56.2. It exists between pH 5.0 and 10.1; it is present alone in the pH-ranges 5.0–7.7 and 8.9–9.4, and together with component F in the pH-range 7.7–8.9, and together with component I in the pH-range 9.4–10.1. Component F is very inhomogeneous and present in small quantity, therefore its sedimentation constant is not well-defined. In one run the value 40.9 was found. Component I appears first in small quantity

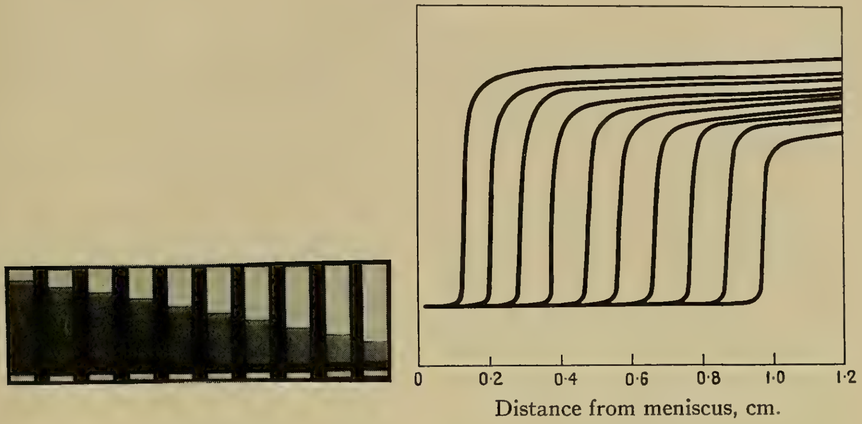


FIG. 29*a*. FIG. 29*b*.

FIG. 29. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Loligo vulgaris* at pH 6.8 (s_{20} = 56.7); centrifugal force 60,000; time between exposures 5 minutes.

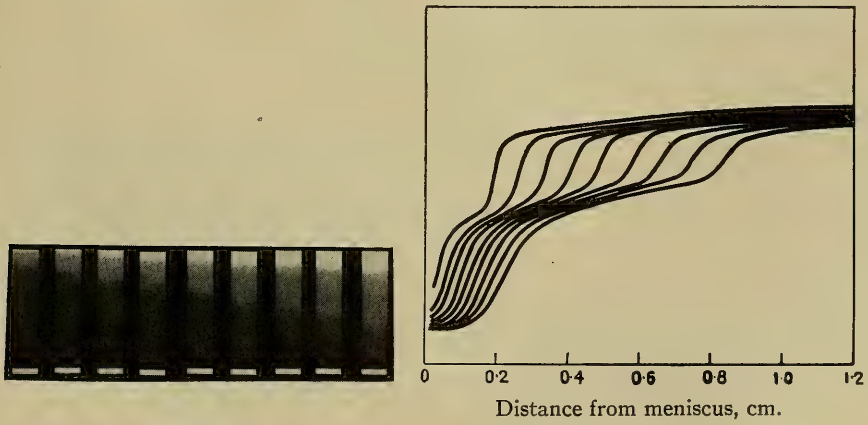


FIG. 30*a*. FIG. 30*b*.

FIG. 30. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Loligo vulgaris* at pH 7.7 (s_{20} = 56.7 and 35.9); centrifugal force 50,000; time between exposures 5 minutes.

together with D, but its relative amount increases with pH. Above pH 10.1 it is present alone. It shows a behavior similar to component I in the *Loligo*-blood. In solutions more acid than pH 5 the hemocyanin is precipitated. Figures 32 and 33 give samples of sedimentation runs and Fig. 34 (Plate IV) the pH-stability curve.

TABLE XIX

Rossia owenii

Dilution of blood 15 times; centrifugal force below pH 10 60,000 (speed 30,000 r.p.m.); above pH 10 215,000 (speed 55,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	S_{20} D	S_{20} F	S_{20} I
HAc, NaAc, NaCl	5.0	0.24	59.3		
KH_2PO_4, Na_2HPO_4, NaCl	5.5	0.24	56.0		
" " "	5.9	0.24	55.0		
" " "	6	0.24	53.7		
" " "	6.4	0.24	52.9		
" " "	6.8	0.24	57.0		
" " "	7.4	0.24	53.8		
" " "	7.7	0.24	57.7	+	
" $Na_2B_4O_7$, "	8.4	0.30	56.4	+	
" " "	8.9	0.30	57.5	40.9	
Na_2CO_3, " "	9.2	0.30	56.5		
" " "	9.4	0.30	56.3		+
" " "	9.8	0.30	58.8		+
" " "	10.1	0.30	59.0		13.0
" " "	10.3	0.30			11.4
" " "	10.6	0.30			10.4
NaOH, Na_2HPO_4, NaCl	11.1	0.39			10.3
" " "	11.5	0.47			10.4
" " "	11.7	0.40			10.0
			56.2		10.9

Sepia officinalis

Roscoff, France

The sedimentation picture of this blood shows two components, D and H, in the pH-range 4.0–8.5. D has the sedimentation constant 55.9 whereas the sedimentation constant of H is 18.7. From pH 4 to 7 the proportions between D and G seem to be constant. From the centrifuge curves it can be estimated roughly that the percentage of D is 75, of H 25, assuming that they have the same light absorption. The amount of H rises towards the alkaline side. In the region 8.5–10.4 another component is observed, which is very inhomogeneous and with a sedimentation constant higher than H. In this region the inhomogeneity of the material makes the measurements rather uncertain. At pH 10.6 a homogeneous dissociation product, I, is formed. Its sedimentation constant is originally 11.8 but drops gradually as in the case of *Loligo* and *Rossia.* At pH 4 an increase in the sedimentation constants of D and H shows that aggregation sets in. At 3.6 the

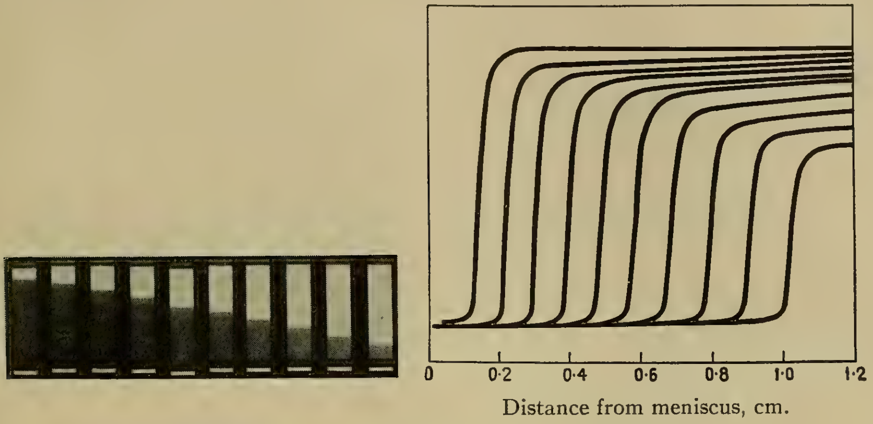


FIG. 32*a*. FIG. 32*b*.

FIG. 32. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Rossia owenii* at pH 6.0 (s_{20} = 56.2); (centrifugal force 70,000; time between exposures 5 minutes.

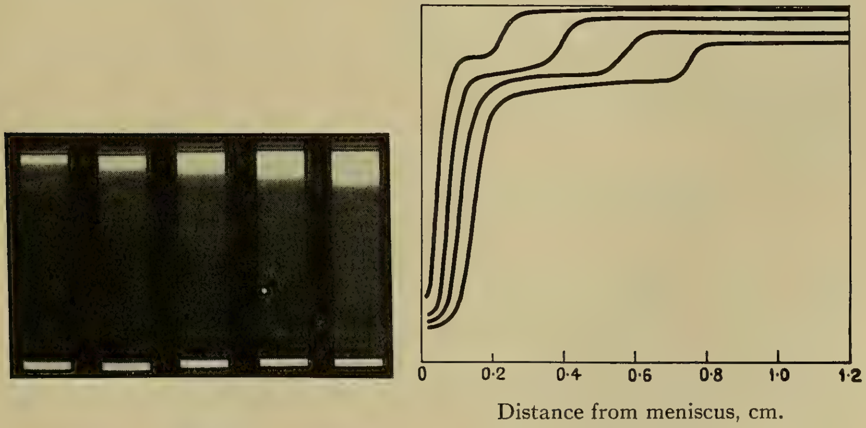


FIG. 33*a*. FIG. 33*b*.

FIG. 33. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Rossia owenii* at pH 10.1 (s_{20} = 56.2 and 13.0); centrifugal force 120,000; time between exposures 5 minutes.

protein is precipitated. Figures 35 and 36 give examples of sedimentation runs, Fig. 37 (Plate IV) the pH-stability diagram.

Octopus vulgaris
Roscoff, France

The main component, E, of this hemocyanin has the sedimentation constant 49.3. It is present in the blood in the pH-region 4.0–10.3.

Table XX

Sepia officinalis

All conditions the same as in Table XVIII.

Solvent	pH of solvent	Total molar	S_{20} D	S_{20} H	S_{20} I	
NaAc, HAc, NaCl.	4.0	0.20	62.6*	27.3*		Inhomogeneous
" " "	4.6	0.20	58.9	23.9		
" " "	5.0	0.20	53.4	18.9		
" " "	5.5	0.20	56.8	15.2		
KH_2PO_4, Na_2HPO_4, NaCl.	6.0	0.20	54.7	20.9		
" " "	6.5	0.20	53.5	18.3		
" " "	6.8	0.20	57.1	17.6		
" " "	6.8	0.20	57.2	+		
" " "	7.4	0.20	55.4	18.3		
" " "	8.0	0.20	49.8	18.9		
" $Na_2B_4O_7$, "	8.5	0.25	54.3	18.5†		
Na_2CO_3, " "	9.0	0.25	55.2	+†		
" " "	9.4	0.25	58.0	20.0*†		
" " "	10.0	0.25	+	20.5*		Inhomogeneous
" " "	10.6	0.25			11.8	
NaOH, Na_2HPO_4, NaCl.	11.1	0.30			10.5	
" " "	11.5	0.30			10.9	
" " "	11.7	0.30			9.1	
			55.9	18.7	10.6	

* Not used for the calculation of mean value.

† Three components present, the third with a sedimentation constant between D and H. At 9.4 the value 42 was obtained.

At pH 9.5 it is dissociated to a small degree into component I. The relative amount of I increases with pH. It appears together with E in the pH-region 9.5–10.3, but above pH 10.3 it is alone. The sedimentation constant at pH 10.3 is 12.1, but above it drops. I seems to be identical with the alkaline dissociation products of the decapods. In the pH region 4.0–9.5 E is generally the only component present; in three runs it was accompanied by a lower component each time with different sedimentation constant. Figure 38 (Plate IV) gives the pH-stability diagram.

The pH-stability diagram has been studied here before (Svedberg and Eriksson, 1932). If the value given there for the sedimentation constant of the main component is corrected for the influence of density and viscosity of the buffer solutions, it becomes 52.6. This agrees fairly well with the present determination. After correction

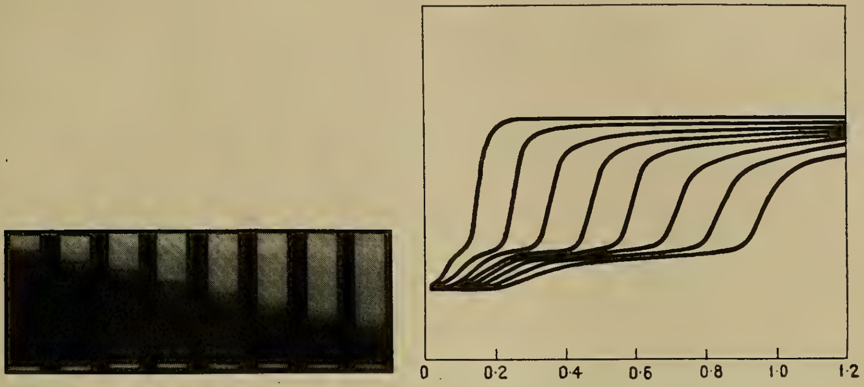


FIG. 35*a*. FIG. 35*b*.

FIG. 35. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Sepia officinalis* at pH 5.5 (s_{20} = 55.4 and 18.7); centrifugal force 71,000; time between exposures 5 minutes.

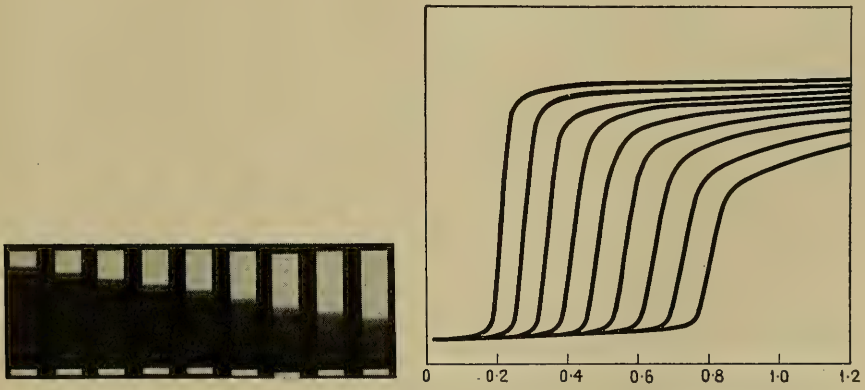


FIG. 36*a*. FIG. 36*b*.

FIG. 36. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Sepia officinalis* at pH 11.5 (s_{20} = 10.6); centrifugal force 220,000; time between exposures 5 minutes.

this value is constant inside the pH-region in which the main component is present. During the earlier investigation the blood was not stored in frozen condition as now and was dialyzed for some days against the buffer instead of being diluted just before the run. This might explain the appearance of a low-molecular component with a sedimentation constant changing with pH. A similar component has only appeared in a few runs in this work.

TABLE XXI

Octopus vulgaris

Dilution of blood 20 times; centrifugal force 100,000 (speed 37,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	S_{20} E	S_{20} I	S_{20}
NaAc, HAc, NaCl	4.0	0.20	51.6		
" " "	4.6	0.20	52.4		
" " "	5.0	0.20	49.9		
Na_2HPO_4, KH_2PO_4, NaCl	5.6	0.20	47.6		
" " "	6.0	0.20	48.8		
" " "	6.3	0.20	48.5		13.7
" " "	6.8	0.20	47.6		
" " "	7.3	0.20	48.0		
" " "	7.7	0.20	48.9		20.1
" " "	8.0	0.20	47.8		15.2
$Na_2B_4O_7$, " "	8.4	0.25	45.7		
" " "	8.8	0.25	45.2		
" Na_2CO_3, "	9.0	0.25	49.5		
" " "	9.3	0.25	51.1		
" " "	9.5	0.25	51.9	+	
" " "	9.8	0.25	52.8	+	
" " "	10.3	0.25	51.4	12.1	
" " "	10.8	0.25		11.3	
			49.3		

Eledone moschata

Naples, Italy

The main component E of the hemocyanin is present from pH 5 to 11.0. The sedimentation constant is 49.1. At 10.6 about ten per cent of the protein splits up into smaller molecules having the sedimentation constant of 11.8 (component I). At 11.0 the percentage of the lighter protein goes up to 50. At pH 11.1 the splitting is complete. The sedimentation constant of the very uniform dissociation product drops with increasing pH. At pH 5.4 the protein is very inhomogeneous. In more acid solutions precipitation sets in.

Figures 39 and 40 give examples of sedimentation runs, Fig. 41 (Plate IV) the pH-stability diagram.

REVERSIBILITY

Some experiments have been carried out on the reversibility of the dissociation reactions and the equilibrium between the dissociation products. Two hemocyanins have been chosen for these tests, viz. the blood pigments of *Helix pomatia* and *Limulus polyphemus*. They can be regarded as representatives for two different groups. In the natural blood and in a large part of the investigated pH-region the *Helix* hemocyanin consists of just one molecular species. The *Limulus* hemocyanin, on the contrary, contains four different components in its natural state and inside the main part of the stability region (from pH 5 to 10.5).

TABLE XXII

Eledone moschata

Dilution of blood 15 times; centrifugal field 75,000 (speed 32,000); in the four last runs 230,000 (speed 57,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	S_{20} E	S_{20} I	
HAc, NaAc, NaCl	5.4	0.20	49.4		Very inhomogeneous
KH_2PO_4, Na_2HPO_4, NaCl	5.9	0.20	49.2		
" " "	6.2	0.20	47.8		
" " "	6.5	0.20	48.7		
" " "	6.8	0.20	48.1		
" " "	7.1	0.20	46.7		
" " "	7.4	0.20	48.5		
" " "	7.7	0.20	49.7		
" " "	8.0	0.20	50.0		
" $Na_2B_4O_7$, "	8.5	0.25	48.2		
Na_2CO_3, " "	9.1	0.25	50.8		
" " "	9.5	0.25	50.3		
" " "	9.8	0.25	49.9		
" " "	10.3	0.25	50.1		
" " "	10.6	0.25	49.8	11.8	
" " "	11.0	0.25	48.2	10.5	
" " "	11.1	0.25		11.4	
NaOH, Na_2HPO_4, NaCl	11.5	0.30		9.46	
" " "	11.5	0.30		9.59	
" " "	11.7	0.30		8.57	
			49.1	10.60	

Helix pomatia

The reversibility of the dissociation caused by a change in pH was studied. The results are summarized in Table XXIII. The blood was taken to pH_I, chosen so that some sort of dissociation took place, and then to pH_{II}; where it normally would have been less dissociated. In Table XXIII are given the values for the sedimentation constants

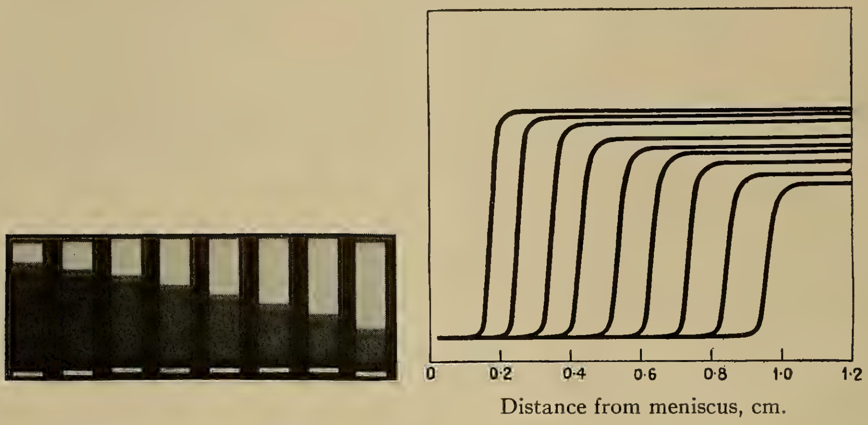


FIG. 39*a*. FIG. 39*b*.

FIG. 39. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Eledone moschata* at pH 9.8 (s_{20} = 49.1); centrifugal force 72,000; time between exposures 5 minutes.

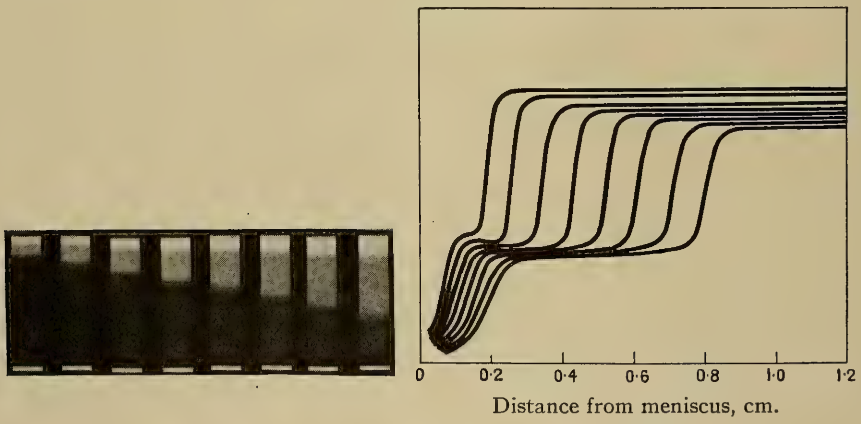


FIG. 40*a*. FIG. 40*b*.

FIG. 40. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Eledone moschata* at pH 11.0 (s_{20} = 49.1 and 10.6); centrifugal force 72,000; time between exposures 5 minutes.

at pH_I, the normal sedimentation constants at pH_{II}, and the sedimentation constants found at pH_{II} when the protein had first been taken to pH_I. The results show that the dissociation is reversible inside a certain pH-region, but if the solution is made too alkaline only a part of the hemocyanin is associated into the normal components. Another part of it aggregates, but into smaller particles than normally found. If the solution is brought to pH 12 only inhomogeneous material is formed by neutralization.

The equilibrium between the components was studied in the following way: a solution at pH 8.0, containing the components B and C, was run in the ultracentrifuge until component B had sedimented to the bottom of the cell. The centrifuge was stopped and the liquid pipetted off. The sediment was stirred up in a small quantity of the same buffer. Runs were made on these two solutions. The initial solution contained 67 per cent B and 33 per cent C, the solution pipetted off 73 per cent C and 27 per cent B and the sediment 67 per cent C and 33 per cent B. These figures agree within limits of error. The relative amounts are obtained from the microphotometric curves of the sedimentation pictures assuming that the different components have the same light absorption. The dissociation products seem to be in equilibrium. If the relative concentration of one is diminished the same proportions are reëstablished.

TABLE XXIII

Helix pomatia

pH_I	pH_{II}	s_{20}	At pH_I		s_{20} for comp. normal at pH_{II}		s_{20} for comp. found at pH_{II}			
8.0	6.8	98.9	62.0	16.0	98.9		95.7			
8.9	6.8			16.0	98.9		99.0			
8.6	7.7			16.0	98.9	62.0		60.7	17.9	Inhomogeneous
9.5	5.9		16.0	12.1	98.9		106.8	66.1	22.3	
10.3	5.0		16.0	12.1	98.9		94.1	63.4	23.0	
10.5	6.8		16.0	12.1	98.9		97.9	64.8	26.2	
10.8	5.9		16.0	12.1	98.9		92.8	60.1	22.7	
10.8	7.7		16.0	12.1	98.9	62.0	+	59.8	+	
10.8	5.9		16.0	12.1	98.9		109.8	70.5	23.6	
12.1	5.9		Inhomogeneous		98.9			32.3		Inhomogeneous
4.0	6.8	98.9	62.0		98.9		85.7			Two components, bad separation
4.0	5.4	98.9	62.0		98.9		100.9			

Limulus polyphemus

The reversibility on changing the pH was studied at pH 10.8, where the protein is dissociated into a product with sedimentation constant 5.87, and at 4.0, where it contains three components. The dissociation reactions were completely reversible.

In order to study the equilibrium between the components the same procedure as in the case of the *Helix* hemocyanin was carried out. A solution containing the four components D, F, H, and K was centrifuged until D had reached the bottom. In the initial solution the composition was:

22 per cent D, 14 per cent F, 41 per cent H, and 23 per cent K.

In the pipetted-off solution

14 per cent D, 11 per cent F, 55 per cent H, and 20 per cent K.

In the sediment

58 per cent D, 17 per cent F, 25 per cent of H and K together.

Evidently the sediment contains a larger percentage of the heavy component D and less of the light components H and K, while the supernatant solution contains less of D and more of H and K.

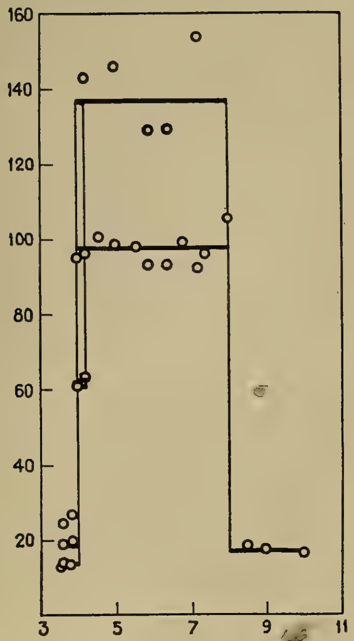
The solutions were stored for a week and run again. The values then obtained agreed with those given above within the limits of error. The components therefore do not seem to be in equilibrium in this case.

If the components are in equilibrium the equilibrium must be disturbed during the ordinary runs. The concentration of the higher components in the solution decreases faster than the concentration of the lower components. Whether or not this shows in the sedimentation diagrams must depend on the rate of reëstablishment of the equilibrium and the time taken by the run. In some cases such an effect has been observed.

EXPLANATION OF PLATE IV

pH-stability diagrams for hemocyanins of species listed below. Abscissæ in all figures, pH; ordinates, s_{20}.

FIG. 28. *Limax maximus.*
FIG. 31. *Loligo vulgaris.*
FIG. 34. *Rossia owenii.*
FIG. 37. *Sepia officinalis.*
FIG. 38. *Octopus vulgaris.*
FIG. 41. *Eledone moschata.* The dotted line in Fig. 41 indicates the position of the isoelectric point.



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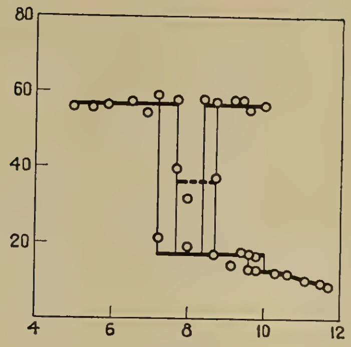
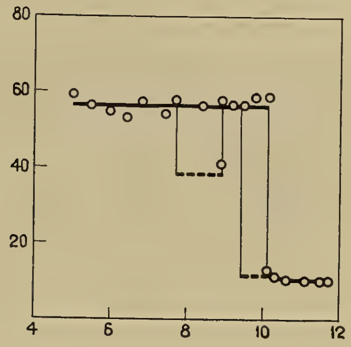
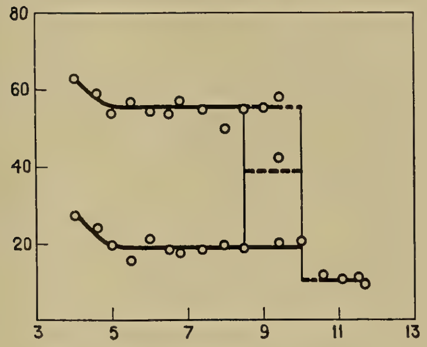


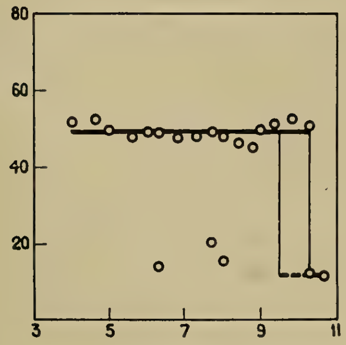
31



34



37



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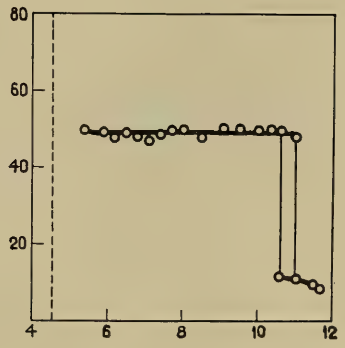

41

PLATE IV

MOLECULAR WEIGHTS

A number of sedimentation equilibrium measurements have been carried out so as to make it possible to coördinate sedimentation constants and molecular weights. In cases where more than one molecular species is present the computation of molecular weights from equilibrium data is uncertain and we have therefore refrained

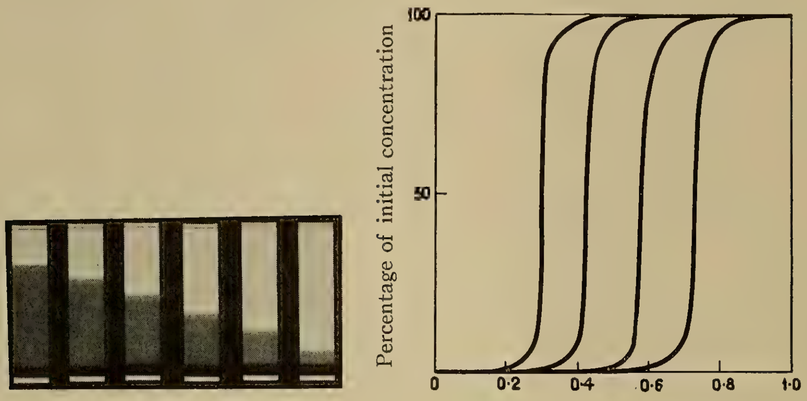

FIG. 42*a*. FIG. 42*b*.

FIG. 42. Sedimentation pictures (*a*) and concentration curves (*b*) for hemocyanin from *Helix pomatia* at pH 5.5 ($s_{20} = 98.9$); centrifugal force 45,000; time between exposures 5 minutes.

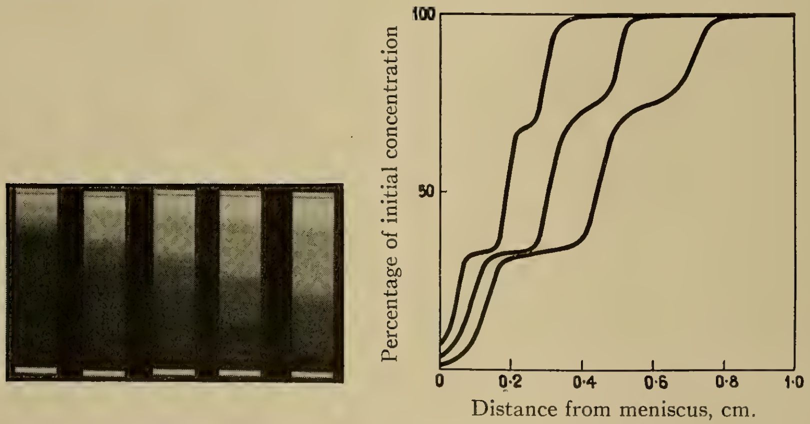

FIG. 43*a*. FIG. 43*b*.

FIG. 43. Sedimentation pictures (*a*) and concentration curves (*b*) for hemocyanin from *Helix pomatia* at pH 8.2 ($s_{20} = 98.9$, 62.0, and 16.0); centrifugal force 60,000; time between exposures 5 minutes.

from studying solutions of this kind. The results are summarized in Table XXIV.

DISCUSSION OF RESULTS

The most striking result of this investigation is perhaps the perfect homogeneity of the various hemocyanins with regard to molecular weight. Not only the main component or components which exist in the region comprising the isoelectric point (Fig. 42) but also the products of reversible dissociation (Fig. 43) are definite molecular individuals. At very low and very high pH-value where irreversible dissociation occurs the products are inhomogeneous (Fig. 44). Compare with Fig. 45.

TABLE XXIV

Sedimentation equilibrium determinations on hemocyanins

Species	Dilution of blood	Solvent	Total molar	pH	Thickness of cell	r.p.m.	Method	Time in hours	M	M Mean
Pandalus borealis	7	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	4	3800	Scale	160	408000	397000
	7	" " "	0.20	6.8	4	3800	Scale	132	386000	
Palinurus vulgaris	10	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	12	3330	Slit	120	485000	447000
	10	" " "	0.20	6.8	12	3060	Scale	112	462000	
	10	" " "	0.20	6.8	12	3540	Scale	154	450000	
	10	" " "	0.20	6.8	12	3300	Scale	131	409000	
	10	" " "	0.20	6.8	12	3300	Scale	117	429000	
Homarus vulgaris	7	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	12	3700	Slit	137	793000	803000
	7	" " "	0.20	6.8	12	3700	Slit	112	798000	
	7	" " "	0.20	6.8	4	3700	Scale	214	817000	
Calocaris macandræ	4	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	4	2600	Scale	249	1.306000	1.329000
	4	" " "	0.20	6.8	4	2700	Scale	226	1.352000	
Helix pomatia	20	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	12	1500	Slit	168	6.520000	6.680000
	60	" " "	0.20	6.8	12	1500	Slit	208	6.658000	
	120	" " "	0.20	6.8	12	1620	Scale	156	6.864000	
	20	KH_2PO_4, $Na_2B_4O_7$, NaCl	0.25	8.5	4	3180	Slit	161	819000	797000
	20	" " "	0.25	8.5	4	3162	Scale	238	774000	

The components of highest molecular weight always exist in a region comprising the isoelectric point. As a rule the number of components increase from the isoelectric point towards the alkaline and acid side. Among the 22 species studied 13 have only one component at the isoelectric point, 7 have two, 1 three and 1 four components.

The sedimentation constants observed may be arranged in ten classes (A–K), as shown by Table XXV. For each class the variations are almost within the limit of experimental error. To a certain molecular weight may correspond more than one sedimentation constant according to the more or less pronounced deviation from the spherical shape which the molecule shows (Table XXVI). The molecular weights of the hemocyanin found in the blood of a certain species are always simple multiples of the lowest well-defined compo-

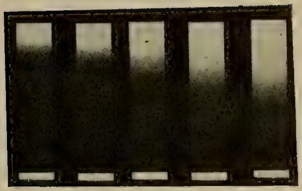

FIG. 44*a*.

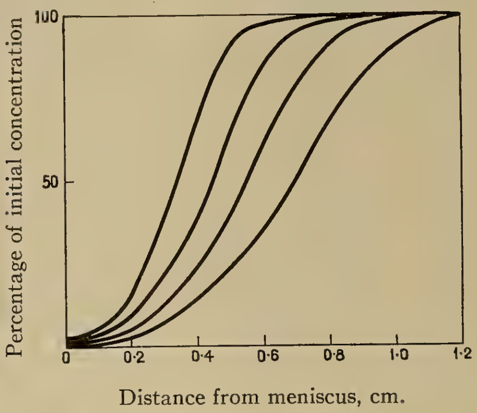

FIG. 44*b*.

FIG. 44. Sedimentation pictures (*a*) and concentration curves (*b*) for hemocyanin from *Nephrops norvegicus* at pH 11.7 (s_{20} = 8.95); centrifugal force 220,000; time between exposures 10 minutes.

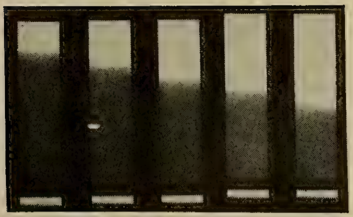

FIG. 45*a*.

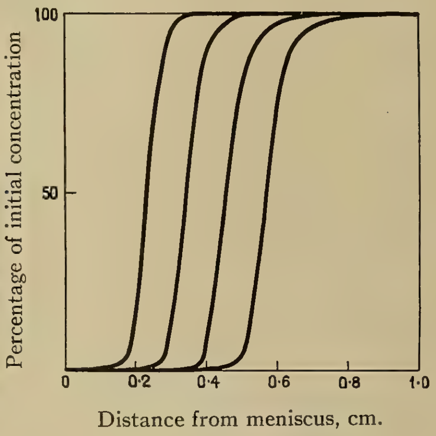

FIG. 45*b*.

FIG. 45. Sedimentation pictures (*a*) and concentration curves (*b*) for hemocyanin from *Nephrops norvegicus* at pH 4.5 (s_{20} = 24.5); centrifugal force 150,000; time between exposures 5 minutes.

nent. Thus for the Malacostraca the relationship is 1 : 2 and for the Gastropoda 2 : 8 : 16 : 24. Moreover, the weights of all the well-defined hemocyanin molecules seem to be simple multiples of the lowest one among them (Table XXVI).

In most cases the hemocyanin components in the blood of a certain species are interconnected by reversible, pH-influenced dissociation-association reactions. At certain critical pH values a profound change in the number and percentage of the components takes place. The shift in pH necessary to bring about the reaction is not more than a few tenths of a unit. Consequently the forces holding the dissociable parts of the molecule together must be very feeble. This fact makes it all the more surprising that an equal backward shift in pH does

TABLE XXV

Sedimentation constants arranged in classes

Species	A	B	C	D	E	F	G	H	I	K
CRUSTACEA:										
Malacostraca										
Pandalus borealis							22.9	17.4		+
Palinurus vulgaris								16.4		4.10
Nephrops norvegicus							24.5	17.1		5.97
Homarus vulgaris							22.6	16.1		+
Astacus fluviatilis							23.3	16.3		4.02
Carcinus mœnas							23.3	16.7		+
Cancer pagurus						32.7	23.6	16.4		4.71
ARACHNOMORPHA:										
Xiphosura										
Limulus polyphemus				56.6		34.6	24.0	16.1		5.87
CONCHIFERA:										
Gastropoda										
Littorina littorea		99.7	63.3					15.0		
Neptunea antiqua		104.0						14.3	+	
Buccinum undatum		102.1	63.8						+	
Busycon canaliculatum	130.4	101.7	61.1					13.5		
Helix pomatia		98.9	62.0					16.0	12.1	
" *arbustorum*		91.2	64.1					15.0	11.1	
" *nemoralis*		101.0	65.0					16.6	11.9	
" *hortensis*		100.0	61.9					15.9	12.1	
Limax maximus	136.1	97.3	61.9				25.5	18.0	13.7	
Cephalopoda										
Decapoda										
Loligo vulgaris				56.7		35.9		16.9	12.1	
Sepia officinalis				55.9		+		18.7	10.6	
Rossia owenii				56.2		+			10.9	
Octopoda										
Octopus vulgaris					49.3				+	
Eledone moschata					49.1				10.6	

really cause an association of the parts to exactly the same molecule. For this reason it is justified, we think, to use the term molecule for the hemocyanin particles despite their enormous size. Their mass cannot be changed continually as in the case of an ordinary colloid particle and they have a finite stability range with regard to the surroundings. The hemocyanin particles, therefore, most probably possess a definite structure.

Table XXVI

Species	pH	S_{20}	D_{20}	M_S	M_e	$M_{calc.}$
Pandalus borealis	6.8	17.4			397,000	
Palinurus vulgaris	6.8	16.4	3.4	446,000	447,000	
Helix pomatia	9.7	12.1	2.23	502,000		1×406,000
Busycon canaliculatum	9.6	13.5	3.29	379,600		=12×33,800
Eledone moschata	11.6	10.6	2.16	457,000		
Nephrops norvegicus	6.8	24.5	2.79	812,000		
Homarus vulgaris	6.8	22.6	2.78	752,000	803,000	2×406,000
Helix pomatia	8.6	16.0	2.06	719,000	797,000	=812,000
Helix nemoralis	8.6	16.6	1.92	799,000		
Calocaris macandræ	6.8	34.0			1,329,000	4×406,000 =1,624,000
Octopus vulgaris	6.8	49.3	1.65	2,785,000		7×406,000
Eledone moschata	6.8	49.1	1.64	2,791,000		=2,842,000
Rossia owenii	6.8	56.2	1.58	3,316,000		8×406,000 =3,248,000
Helix pomatia	6.8	98.9	1.38	6,630,000	6,680,000	16×406,000
Busycon canaliculatum	6.8	101.7	1.38	6,814,000		=6,496,000
Busycon canaliculatum	6.8	130.4	1.17	9,980,000		24×406,000 =9,744,000

The pH-stability diagrams show a number of regularities indicative of biological kinship. All the Malacostraca give similar diagrams and so do the Gastropoda. The Cephalopoda show two types of diagram, one characteristic of the Decapoda and one of the Octopoda. Only one species of the Xiphosura, viz. the *Limulus*, has been studied. It shows a diagram entirely different from that of the Malacostraca species studied. The placing of *Limulus* among the crustaceans as was formerly done is therefore not supported by our sedimentation analysis of its blood pigment.

Within the large groups mentioned above an attempt at classification based upon the similarity of the stability diagrams would give, roughly, the following result:

Malacostraca: *Pandalus* and *Palinurus*—main comp. s = 16.
Nephrops and *Homarus*—main comp. s = 23.
Astacus, *Cancer* and *Carcinus*—both comp. s = 16 and s = 23 through most of the stability range.

Xiphosura: *Limulus*—four comp. s = 57, 34, 16, and 6 through most of the stability range; at the acid side also comp. s = 23.

Gastropoda: **Pulmonata:**
Helix and *Limax*—comp. s = 100 and 61 on both the alkaline and acid side of the isoelectric point.
Aspidobranchia:
Littorina, *Neptunea*, *Buccinum*, and *Busycon*—comp. s = 61 only on the alkaline side.

Cephalopoda, Decapoda:
Loligo, *Rossia*, and *Sepia*—main comp. s = 57.

Cephalopoda, Octopoda:
Octopus and *Eledone*—main comp. s = 49.

Really striking differences in the stability diagrams only occur for species belonging to different orders. All species of one and the same order have similar diagrams. Within the order Cephalopoda the two sections Decapoda and Octopoda show rather different diagrams, though. From the point of view of the blood sedimentation analysis, therefore, the Decapoda and the Octopoda ought perhaps to be regarded as systematic units of the same magnitude as the Gastropoda, i.e. as different orders within the class Mollusca, having developed as parallel branches from common ancestors far down in the Mollusca cladus. It is interesting to see that there is a notable difference between the diagrams for the species belonging respectively to the suborders Pulmonata and Aspidobranchia.

Similarities between species belonging to the same family or the same genus are sometimes revealed by their stability diagram (e.g. *Nephrops* and *Homarus; Helix pomatia* and *Helix nemoralis*) but species from two different although closely related genera may have very similar diagrams (e.g. *Helix hortensis* and *Limax maximus*).

Among the species studied by us no two have quite identical stability diagrams. It should be possible, therefore, to make a species determination based solely on the stability diagram. In such cases where the stability diagrams are rather similar (e.g. *Helix pomatia* and *Helix nemoralis*) a determination of the isoelectric point would settle the question (5.05 for *Helix pomatia* and 4.63 for *Helix nemoralis*) (Pedersen, 1933).

The expenses of this investigation have been defrayed by grants from the Andersson Foundation, the Astra Co., the Nobel Foundation, the Rockefeller Foundation and the Wallenberg Foundation. Living material of the following species were put at our disposal by the Zoölogical Station at Kristineberg, Sweden, viz. *Pandalus borealis*, *Nephrops norvegicus*, *Carcinus mœnas*, *Buccinum undatum*, *Littorina littorea*, *Neptunea antiqua*, and *Rossia owenii*. For this valuable help we are indebted to Professor E. Lönnberg and Dr. G. Gustafson. Samples of blood from *Loligo vulgaris* and *Eledone moschata* were kindly sent us by Professor R. Dohrn and Dr. L. Califano of the Zoölogical Station at Naples. The *Limulus* and *Busycon* blood was given by Dr. A. C. Redfield of the Oceanographic Institution at Woods Hole, Massachusetts. Blood from *Palinurus vulgaris*, *Sepia officinalis* and *Octopus vulgaris* was collected for us at the Marine Station, Roscoff, France, by Dr. Lindahl. For the permission to make this collection we are indebted to Professor Charles Pérez.

SUMMARY

1. Blood from twenty-two species containing hemocyanin as respiratory pigment has been subjected to a detailed ultracentrifugal study.

2. Sedimentation constants have been determined throughout the pH-stability ranges.

3. In some cases sedimentation equilibrium measurements have been carried out. From these data molecular weight values were obtained and correlated with the sedimentation constants.

4. The various hemocyanins are perfectly homogeneous with regard to molecular weight both at the isoelectric point and in the regions where reversible dissociation or association occurs.

5. The stability range for the components of highest molecular weight always comprises the isoelectric point.

6. Except at extremely low and high pH values the dissociation reactions are reversible.

7. The molecular weights of the different hemocyanins and their reversible dissociation products show a relationship of simple multiples.

8. Species belonging to the same order have similar pH-stability diagrams. Each species has a characteristic diagram by means of which it can be identified. In doubtful cases the isoelectric point may be used as complementary evidence.

REFERENCES

LAMM, O., 1933. *Nature,* **132**: 820.
LAMM, O. AND A. POLSON, 1936. *Biochem. Jour.,* **30**: 528.
PEDERSEN, K. O., 1933. *Koll.-Zeitschr.,* **63**: 277.
SVEDBERG, T., 1925. *Koll.-Zeitschr. Erg.-Band z.,* **26**: 53.
SVEDBERG, T., 1927. *Zeitschr. physikal. Chemie,* **127**: 51.
SVEDBERG, T., 1933. *Jour. Biol. Chem.,* **103**: 311.
SVEDBERG, T., 1934*a*. *Chem. Rev.,* **14**: 1.
SVEDBERG, T., 1934*b*. *Science,* **79**: 327.
SVEDBERG, T., 1934*c*. *Koll.-Zeitschr.,* **67**: 1.
SVEDBERG, T., 1934*d*. *Naturwiss.,* **22**: 225.
SVEDBERG, T. AND E. CHIRNOAGA, 1928. *Jour. Am. Chem. Soc.,* **50**: 1399.
SVEDBERG, T. AND I.-B. ERIKSSON, 1932. *Jour. Am. Chem. Soc.,* **54**: 4730.
SVEDBERG, T. AND A. HEDENIUS, 1933. *Nature,* **131**: 325.
SVEDBERG, T. AND A. HEDENIUS, 1934. *Biol. Bull.,* **66**: 191.
SVEDBERG, T. AND F. F. HEYROTH, 1929*a*. *Jour. Am. Chem. Soc.,* **51**: 539.
SVEDBERG, T. AND F. F. HEYROTH, 1929*b*. *Jour. Am. Chem. Soc.,* **51**: 550.
TISELIUS, A. AND D. GROSS, 1934. *Koll.-Zeitschr.,* **66**: 11.

INDEX

ABRAMOWITZ, A. A. Physiology of the melanophore system in the catfish, Ameiurus, 259.
Abstracts of scientific papers presented at Marine Biological Laboratory, summer of 1936, 386.
Aenoplex smithii, spermatogenesis of, cytological phenomena, 375.
Alcohol, effect on gastrulation in Arbacia, 413.
Ameiurus, physiology of melanophore system, 259.
Arbacia punctulata, cleavage without nuclei, 101.
ARMSTRONG, P. B. Mechanism of hatching in Fundulus heteroclitus (abstract), 407.
Ascidians, excretion in, rôle of blood cells, 249.

BACTERIA, multiplication in different volumes stored sea water, influence of oxygen tension and solid surfaces, 324.
Bacteroids, intracellular, of roaches, 433.
BALLARD, W. W. Observations on lens regeneration in Amblystoma (abstract), 388.
BEAMS, H. W., AND R. L. KING. The effect of ultracentrifuging upon chick embryonic cells, with special reference to the "resting" nucleus and the mitotic spindle, 188.
BEAR, R. S. *See* Schmitt, Bear and Young (abstract), 402.
BISSONNETTE, T. H. Modified sexual photoperiodicity in ferrets, raccoons and quail (abstract), 395.
Blood cells, in excretion, ascidians, 249.
BOWEN, R. E. Sensory hairs of the dogfish ear, 429.
BOZLER, E. The double refraction of smooth muscle (abstract), 402.
BRAMBEL, C. E. *See* Cowles and Brambel, 286.
BROOKS, MATILDA M. The effect of methylene blue on the spectrophotometric picture of hemoglobin, CO-hemoglobin and CN-hemoglobin (abstract), 386.
BURTON, ALAN C. The basis of the principle of the master reaction in biology (abstract), 393.
——, —— —. *See* Sichel and Burton (abstract), 397.

CALCIUM, free, in action of stimulating agents on Elodea cells, 306.
CAMERON, J. A., AND K. O. MILLS. Behavior of frog tadpole epidermal cells during seven successive regeneration periods (abstract), 405.
CAMERON, J. A. *See* Fisher and Cameron (abstract), 404.
Carotenoid metabolism, in surf perches, structure and function of gut in, 217.
CAROTHERS, E. ELEANOR. Cellular behavior in abnormal growths produced by irradiation of grasshopper embryos (abstract), 394.
——, —. ——. Components of the mitotic spindle, 469.
Catfish, melanophore system, physiology of, 259.
CHAMBERS, R. Experimental studies on the oil wetting property of the plasma membrane (abstract), 398.
Chick embryonic cells, effect of ultracentrifuging, with reference to "resting" nucleus and mitotic spindle, 188.
Chromatophores, blue, in some freshwater Protozoa, 492.
Chromosomes, salivary gland, structure as affected by mechanical distortion, 238.
CLARK, E. R., AND ELEANOR L. CLARK. Observations on conditions affecting growth of cells and tissues, from microscopic studies on the living animal (abstract), 405.
CLARK, JEAN M. *See* Mazia and Clark, 306.
CLAUS, PEARL E. *See* Guyer and Claus, 462.

Cleavage without nuclei in Arbacia punctulata, 101.
Clowes, G. H. A. *See* Keltch, Clowes and Krahl (abstract), 399.
——, —. —. —. *See* Krahl, Clowes and Taylor (abstract), 400.
Coates, C. W. *See* Smith and Coates, 282.
Coe, W. R. Environment and sex in the oviparous oyster, Ostrea virginica, 353.
——, —. —. Sequence of functional sexual phases in Teredo, 122.
Coonfield, B. R. Regeneration in Mnemiopsis leidyi Agassiz, 421.
Cowles, R. P., and C. E. Brambel. A study of the environmental conditions in a bog pond with special reference to the diurnal vertical distribution of Gonyostomum semen, 286.

Dan, Katsuma, T. Yanagita and M. Sugiyama. The behavior of the cell surface during cleavage (abstract), 395.
Delamater, Arlene Johnson. The effects of heavy water upon the fission rate and the life cycle of the ciliate, Uroleptus mobilis, 199.
Differentiation, energetics of, 59, 82.
Distribution, diurnal vertical, of Gonyostomum semen in a bog pond, 286.
Dogfish ear, sensory hairs, 429.
Dogfish, reactivation by cutting of severed melanophore nerves, 255.
duBuy, H. G. Separation of the conducting and contractile elements in the retractor muscle of Thyone briareus (abstract), 408.

Ear, dogfish, sensory hairs of, 429.
Egg, membranes and germinal vesicle, Sabellaria vulgaris, 46.
Elodea cells, free calcium in action of stimulating agents on, 306.
Environment and sex in oviparous oyster, Ostrea virginica, 353.
Environment, conditions of, in a bog pond, and diurnal vertical distribution of Gonyostomum semen, 286.
Eriksson-Quensel, Inga-Britta, and The Svedberg. The molecular weights and pH-stability regions of the hemocyanins, 498.
Excretion of inorganic phosphate in sculpin, 360.
——, rôle of blood cells, in ascidians, 249.

Figge, Frank H. J. The effect of some oxidation-reduction indicator dyes (phenol indophenol) on the eyes and pigmentation of normal and hypophysectomized amphibians (abstract), 393.
Fish, Charles J. The biology of Oithona similis in the Gulf of Maine and Bay of Fundy, 168.
Fisher, K. C., and J. A. Cameron. The effect of light on the CO-poisoned embryonic Fundulus heart (abstract), 404.
Fission rate, effects of heavy water on, of Uroleptus mobilis, 199.
Fox, Denis L. *See* Young and Fox, 217.
French, C. S. Efficiency of photosynthesis in purple bacteria (abstract), 394.

Gastrulation, effect of alcohol on, in Arbacia, 413.
George, W. C. The rôle of blood cells in excretion in ascidians, 249.
Germinal vesicle, of egg of Sabellaria vulgaris, 46.
Gier, Herschel T. The morphology and behavior of the intracellular bacteroids of roaches, 433.
Glassman, H. N. *See* Jacobs, Glassman and Parpart (abstract), 401.
Gonyostomum semen, diurnal vertical distribution of, and environmental conditions, 286.
Grafflin, Allan L. Renal function in marine teleosts. IV. The excretion of inorganic phosphate in the sculpin, 360.
Grave, B. H., and J. Smith. Hermaphroditism in Mollusca (abstract), 407.
Gut, structure and function, in surf perches with reference to carotenoid metabolism, 217.
Guyer, M. F., and Pearl E. Claus. Recovery changes in transplanted anterior pituitary cells stratified in the ultra-centrifuge, 462.

Hairs, sensory, of dogfish ear, 429.
Hartline, H. K. *See* Lucké, Ricca and Hartline (abstract), 397.

——, —. —. The discharge of impulses in the optic nerve fibers of the eye of Pecten irradians (abstract), 403.

Harvey, Ethel Browne. Parthenogenetic merogony or cleavage without nuclei in Arbacia punctulata, 101.

Harvey, E. N. *See* Hunter and Harvey (abstract), 396.

Heavy water, effects on fission rate of Uroleptus mobilis, 199.

Heilbrunn, L. V. Protein lipid binding in protoplasm, 299.

Hemocyanins, molecular weights and pH-stability regions of, 498.

Henshaw, P. S. The question of recovery from X-ray effects in Arbacia sperm (abstract), 399.

Hörstadius, S. Investigations on determination in the early development of Cerebratulus (abstract), 406.

Hunninen, A. V., and R. Wichterman. Hyperparasitism: A species of Hexamita (Protozoa, Mastigophora) found in the reproductive systems of Deropristis inflata (Trematoda) of marine eels (abstract), 409.

Hunter, F. R., and E. N. Harvey. The effect of lack of oxygen on the permeability of the egg of Arbacia punctulata (abstract), 396.

Hunter, Laura N. Some nuclear phenomena in the Trichodina (Protozoa, Ciliata, Peritrichida) from Thyone briareus (Holothuroidea) (abstract), 406.

Irving, Laurence. Physiological adjustments to diving in the beaver (abstract), 391.

Jacobs, M. H. *See* Parpart and Jacobs (abstract), 400.

——, —. —., H. N. Glassman and A. K. Parpart. Further studies on specific physiological properties of erythrocytes (abstract), 401.

Keil, Elsa M., and F. J. M. Sichel. The injection of aqueous solutions, including acetylcholine, into the isolated muscle fiber (abstract), 402.

Kelley, Robert W. *See* Saxton, Schmeckebier and Kelley, 453.

Keltch, Anna K., G. H. A. Clowes and M. E. Krahl. The respiratory effects exerted by certain organic compounds in relation to their molecular structure (abstract), 399.

Kindred, J. E. An interpretation of the secondary lymphoid nodules in the albino rat (abstract), 390.

King, R. L. *See* Beams and King, 188.

Koonz, Carl H. Some unusual cytological phenomena in the spermatogenesis of a haploid parthenogenetic hymenopteran, Aenoplex smithii (Packard), 375.

Kopac, M. J. Interfacial films between oil and cytoplasm (abstract), 398.

Krahl, M. E. *See* Keltch, Clowes and Krahl (abstract), 399.

——, —. —., G. H. A. Clowes and J. F. Taylor. Action of metabolic stimulants and depressants on cell division at varying carbon dioxide tensions (abstract), 400.

Lackey, J. B. Some freshwater Protozoa with blue chromatophores, 492.

Lipman, Harry J. The phosphatase content of the developing chick embryo (abstract), 386.

Lucas, Alfred M. Nerve cells without central processes in the fourth spinal ganglion of the frog (abstract), 387.

Lucké, B., R. Ricca and H. K. Hartline. Comparative permeability to water and certain solutes of the egg cells of three marine invertebrates (Arbacia, Cumingia and Chaetopterus) (abstract), 397.

MacDougall, Mary S. Cytological studies of the genus Chilodonella, III. The conjugation of Chilodonella labiata, variation? (abstract), 410.

Marine Biological Laboratory, thirty-eighth report, 1.

Martin, Earl A. Asexual reproduction in Dodecaceria fimbriatus (abstract), 396.

Mazia, Daniel, and Jean M. Clark. Free calcium in the action of stimulating agents on Elodea cells, 306.

Melanophore nerves, reactivation by cutting, in dogfish, 255.

—— system, physiology of, in catfish, 259.

Melanosis, cutaneous, in lungfishes, 282.
Membranes and germinal vesicle of egg of Sabellaria vulgaris, 46.
Merogony, parthenogenetic, or cleavage without nuclei in Arbacia punctulata, 101.
Metz, C. W. Effects of mechanical distortion on the structure of salivary gland chromosomes, 238.
Mills, K. O. *See* Cameron and Mills (abstract), 405.
Mitotic spindle, components of, 469.
—— ——, effect of ultracentrifuging on, in chick embryonic cells, 188.
Mnemiopsis leidyi, regeneration in, 421.
Molecular weights and pH-stability regions of the hemocyanins, 498.
Mosquito larvae, utilization of solutes by, 343.
Mustelus, reactivation by cutting of severed melanophore nerves, 255.

NITRITE in the sea, occurrence and significance of, 133.

OITHONA similis, biology of, in Gulf of Maine and Bay of Fundy, 168.
Oyster, environment and sex in, 353.
Oxygen tension, influence on multiplication of bacteria, 324.

PARKER, G. H. The reactivation by cutting of severed melanophore nerves in the dogfish, Mustelus, 255.
Parpart, A. K. *See* Jacobs, Glassman and Parpart (abstract), 401.
——, —. —. The permeability of the erythrocytes of the ground-hog (abstract), 387.
——, —. —., and M. H. Jacobs. Paradoxical osmotic volume changes in erythrocytes (abstract), 400.
Parthenogenetic merogony, or cleavage without nuclei, in Arbacia punctulata, 101.
Phosphate, inorganic, excretion of, in sculpin, 360.
pH-stability regions of the hemocyanins, 498.
Pituitary, anterior, cells, recovery changes in, after stratification in ultra-centrifuge, 462.
Program and abstracts of scientific papers presented at the Marine Biological Laboratory in the summer of 1936, 386.
Protein lipid binding in protoplasm, 299.

RAKESTRAW, Norris W. The occurrence and significance of nitrite in the sea, 133.
Reactivation by cutting of severed melanophore nerves in dogfish, 255.
Recovery changes in transplanted anterior pituitary cells stratified in the ultra-centrifuge, 462.
Regeneration in Mnemiopsis leidyi Agassiz, 421.
Renal function in marine teleosts: excretion of inorganic phosphate in sculpin, 360.
Ricca, R. *See* Lucké, Ricca and Hartline (abstract), 397.
Riethmiller, Grace. *See* Wolf and Riethmiller (abstract), 391.
Roaches, intracellular bacteroids of, 433.
Rugh, Roberts. A quantitative analysis of the anterior pituitary-ovulation relation in the frog (abstract), 389.

SABELLARIA vulgaris, membranes and germinal vesicle of egg of, 46.
Salivary gland chromosomes, effects of mechanical distortion on structure of, 238.
Sandow, Alexander. Diffraction patterns of striated muscle and sarcomere behavior during contraction (abstract), 392.
Saslow, G. Prevention of edema in frog perfusions in the absence of serum proteins (abstract), 404.
Saxton, John A., Mary M. Schmeckebier and Robert W. Kelley. Autogenous transplantations of pigmented and unpigmented ear skin in guinea pigs, 453.
Schechter, Victor. Comparative hypotonic cytolysis of several types of invertebrate egg cells and the influence of age (abstract), 410.
Schmeckebier, Mary M. *See* Saxton, Schmeckebier and Kelley, 453.
Schmitt, F. O., R. S. Bear and J. Z. Young. Some physical and chemical properties of the axis cylinder of the giant axons of the squid, Loligo pealii (abstract), 402.
Schoepfle, G., and J. Z. Young. The structure of the eye of Pecten (abstract), 403.
Sculpin, excretion of inorganic phosphate, 360.

Sex and environment in the oviparous oyster, Ostrea virginica, 353.
Sexual phases, functional, sequence of, in Teredo, 122.
Sichel, F. J. M. *See* Keil and Sichel (abstract), 402.
——, —. —. —., and A. C. Burton. A kinetic method of studying surface forces in the egg of Arbacia (abstract), 397.
Smith, G. M., and C. W. Coates. Cutaneous melanosis in lungfishes (Lepidosirenidae), 282.
Smith, J. *See* Grave and Smith (abstract), 407.
Solutes, utilization by mosquito larvae, 343.
Speidel, C. C. Experiments on the contractile substance of muscle fibers (abstract), 401.
Spermatogenesis, cytological phenomena of a haploid parthenogenetic hymenopteran, Aenoplex smithii (Packard), 375.
Steinbach, H. Burr. Effects of salts on the injury potentials of frog's muscle (abstract), 388.
Stimulating agents, free calcium in action of, on Elodea cells, 306.
Stunkard, H. W. Notes on life cycles of digenetic trematodes (abstract), 411.
Sugiyama, M. *See* Dan, Yanagita and Sugiyama (abstract), 395.
Svedberg, The. *See* Eriksson-Quensel and Svedberg, 498.

Taylor, J. F. *See* Krahl, Clowes and Taylor (abstract), 400.
Teredo, sequence of functional sexual phases, 122.
Trager, William. The utilization of solutes by mosquito larvae, 343.
Transplantations, autogenous, of pigmented and unpigmented ear skin in guinea pigs, 453.
Tyler, Albert. On the energetics of differentiation, III, IV, 59, 82.

Ultra-centrifuge, recovery changes in transplanted anterior pituitary cells stratified in, 462.
——, effect on chick embryonic cells, with reference to "resting" nucleus and mitotic spindle, 188.
Uroleptus mobilis, effect of heavy water on fission rate and life cycle, 199.

Waterman, A. J. Inhibition of gastrulation in Arbacia with nickel chloride (abstract), 411.
——, —. —. The effect of alcohol on gastrulation in Arbacia, 413.
——, —. —. The membranes and germinal vesicle of the egg of Sabellaria vulgaris, 46.
Wichterman, R. Division and conjugation in Nyctotherus cordiformis (Ehr.) Stein (Protozoa, Ciliata) with special reference to the nuclear phenomena (abstract), 412.
——, —. *See* Hunninen and Wichterman (abstract), 409.
Wolf, E. Alfred, and Grace Riethmiller. Studies in calcification: III. The shell of the hen's egg (abstract), 391.

Yanagita, T. *See* Dan, Yanagita and Sugiyama (abstract), 395.
Young, J. Z. *See* Schmitt, Bear and Young (abstract), 402.
——, —. —. *See* Schoepfle and Young (abstract), 403.
——, Robert T., and Denis L. Fox. The structure and function of the gut in surf perches (Embiotocidae) with reference to their carotenoid metabolism, 217.

Zobell, Claude E. Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces, 324.

MARINE
BIOLOGICAL LABORATORY
SUPPLY DEPARTMENT

LIVING MARINE MATERIAL

For a number of years the Supply Department has been furnishing Living Marine Materials. From experience it has been found that during the period between the first of November and the end of February these live animals and plants can be shipped with the most success. This service has proven to be of great value to both instructor and student, particularly to those who are located at some distance from the sea coast.

MARINE AQUARIA SETS

The following marine sets will be supplied during November, December, January, and February. **We assume responsibility for shipments going as far west as the Mississippi and as far south as Georgia, and guarantee to deliver the animals in good condition.** Although we have been successful in shipping live Marine Aquaria Sets to New Mexico, Texas, Missouri, North Dakota, and Iowa, we do not guarantee safe delivery except as stated above.

Set I. Live Marine Material for five-gallon Aquarium. Includes the following:

5 gallons Sea water
2 small Metridium (Sargartia may be substituted)
2 small Starfish
1 Hermit Crab
2 small Sea Urchins
1 small Thyone
2 small Mytilus
2 Littorina
Green algae (Ulva when available)

Price per set **$5.00**

Set II. Live Marine Material for ten-gallon Aquarium. Includes the following:

10 gallons Sea water
4 small Metridium
2 small Starfish
1 Red Starfish (Henricia)
Green algae (Ulva when available)
3 small Sea Urchins
2 small Thyone
2 small Mytilus
Balanus
2 Hermit Crabs
Littorina or Urosalpinx
1 Nereis
1 small Limulus
Astrangia
1 Brittle Starfish

Price per set **$10.00**

Other marine animals not listed can be supplied on short notice. Substitutions can be made on any of the above sets. Prices on larger sets will be gladly given upon request.

All shipping containers furnished at cost. Credit of $1.50 will be given for each carboy returned by prepaid express.

The sea water for these sets is shipped a day or so in advance of the materials, together with instructions for the care of the living forms, in order that the aquarium may be set up and ready for the specimens when they arrive.

If there is any further information desired regarding the above sets, we are very glad to furnish same.

MARINE BIOLOGICAL LABORATORY
WOODS HOLE, MASS.

CONTENTS

Page

WATERMAN, A. J.

The Effect of Alcohol on Gastrulation in Arbacia 413

COONFIELD, B. R.

Regeneration in Mnemiopsis leidyi, Agassiz 421

BOWEN, R. E.

Sensory Hairs of the Dogfish Ear 429

GIER, HERSCHEL T.

The Morphology and Behavior of the Intracellular Bacterioids of Roaches 433

SAXTON, JOHN A., JR., MARY M. SCHMECKEBIER AND ROBERT W. KELLEY

Autogenous Transplantations of Pigmented and Unpigmented Ear Skin in Guinea Pigs 453

GUYER, M. F., AND PEARL E. CLAUS

Recovery Changes in Transplanted Anterior Pituitary Cells Stratified in the Ultra-Centrifuge 462

CAROTHERS, E. ELEANOR

Components of the Mitotic Spindle with Especial Reference to the Chromosomal and Interzonal Fibers in the Acrididæ 469

LACKEY, JAMES B.

Some Fresh Water Protozoa with Blue Chromatophores 492

ERIKSSON-QUENSEL, INGA-BRITTA, AND THE SVEDBERG

The Molecular Weights and pH-Stability Regions of the Hemocyanins 498

INDEX 548

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